Understanding the molecular response of potato during interactions with *Pectobacterium*

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Pectobacterium atrosepticum (*Pba* SCRI1043) and *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbr* ICMP19477) are considered necrotrophic/hemibiotrophic bacterial plant pathogens belonging to the *Enterobacteriaceae* family. They are responsible for blackleg disease on potato stems during the growing season and soft rot of tubers post-harvest. Coronafacic acid (CFA) is a virulence determinant in these *Pectobacterium* taxa, supporting the development of blackleg in potato stems, but little is known about its role during infection of potato tubers or the mechanisms associated with its activity. As *Pba* and *Pbr* are seed borne pathogens whose spread is primarily dependent on contaminated tubers, determining the molecular interactions that occur between pathogen and host in potato tubers and understanding the role of important virulence factors such as CFA in this process, are vital to implementing effective control strategies. In this study, RNA sequencing technology was used to examine the transcriptional response in susceptible potato tubers ('Summer Delight') challenged with *Pba* SCRI1043 or *Pbr* ICMP19477. In addition, to begin to understand the function of CFA, the transcriptional response of the host to *Pba* SCRI1043 was compared to that elicited upon infection with a mutant unable to produce CFA.

In the first part of the study, the most appropriate software available for detection of differential expression in potato was established by comparing the quality of the differential expression data produced by two software packages, DESeq2 and Cuffdiff2. Comparison of the read counts and fold change data from both software packages confirmed the detection of fewer false positives using DESeq2. Based on this finding, DESeq2 was chosen as the most

robust approach for differential expression detection. Subsequent comparison of the potato tuber transcriptome following infection by either *Pbr* ICMP19477 or *Pba* SCRI1043 revealed that the number of differentially expressed genes was consistently higher across 6, 12 and 24 hours post inoculation in response to *Pbr* ICMP19477. Furthermore, the magnitude of the transcriptional change (as determined by log2 fold change values) observed in potato tubers was consistently higher upon inoculation with *Pbr* ICMP19477 than in response to *Pba* SCRI1043. Consistent with the production of plant cell wall degrading enzymes by both pathogens, infection with *Pbr* ICMP19477 and *Pba* SCRI1043 induced a characteristic damage associated molecular patterns-mediated pattern-triggered immunity (PTI) defence response. In particular, genes involved in the early PTI defence response including the PI-PLC signalling pathway, oxidative burst and ethylene (ET) biosynthesis and signalling were significantly differentially expressed. Secondary metabolism was also induced; genes involved in the synthesis of terpenoids showing significant up regulation in response to both soft rot *Enterobacteriaceae* (SRE). SRE infection also led to up regulation of genes related to phytoalexins biosynthesis, including divinyl ether synthase, which is involved in the synthesis of the antimicrobials colneleic and colnelenic acid. Interestingly, no differential expression of genes relating to jasmonic acid (JA) biosynthesis was observed, although the genes encoding *MYC2* and *JAZ*, related to downstream JA signalling, were significantly induced indicative of JA-independent *MYC2* activation. *Pbr* ICMP19477 appeared to elicit additional specific responses by the host, with the two snakin genes *Sn-1* and *Sn-2* amongst those genes with greatest up regulation in response to this pathogen. *Sn-1* and *Sn-2* encode well known antimicrobial peptides with activity against SRE. A specific cluster of LRR genes also seemed to be up regulated in response to pectobacteria, indicative of the host initiating an effector-triggered immunity during the susceptible interaction with this pathogen.

In *Pba* SCRI1043 and *Pbr* ICMP19477, the biosynthetic gene clusters responsible for CFA biosynthesis are harboured on closely related horizontally acquired islands (HAIs). Interestingly, loss of the HAI (HAI2) in *Pba* SCRI1043 resulted in very few observable changes in differential expression in potato tubers, even though the CFA cluster it harbours enhances infection of the stem. The notable exception to this general lack of differential expression when compared to wild type was the expression of *MYC2*. Specifically, no differential expression of *MYC2* was observed upon infection with the CFA mutant when compared to the non-inoculated control whereas *MYC2* was induced in response to *Pba* SCR1043 (and *Pbr*

ICMP19477). This result suggested that CFA might target the *MYC2*-mediated JA response, which alters the production of key defence-related products required to combat bacterial infection. This is consistent with the targeting of the MYC2-JAZ complex by coronatine, a related toxin produced by taxa of *Pseudomonas syringae*.

To study how CFA might influence virulence further, potato tubers were also exposed to exogenous CFA and their transcriptional response was subsequently compared to those of the tubers treated with SRE or the non-inoculated control. These comparisons showed a significant overlap in differential expression upon exposure to CFA and the SRE, with CFA impacting 40% of the genes that responded to inoculation with the bacteria. In particular, application of exogenous CFA resulted in differential expression of the JA and ET signalling pathways in much the same way as inoculation with *Pba* SCRI1043 and *Pbr* ICMP19477. In addition, *JAR1* was up regulated in response to exogenous CFA at 6 hpi. *JAR1* encodes JA-amino acid synthase, an enzyme involved in the conjugation of JA to amino acids. Given that CFA is a molecular mimic of jasmonate, this result suggested that the presence of CFA induces its conjugation to an unknown amino acid. It is probable that the conjugate is necessary for downstream activation of JA signalling and, in particular, differential expression of *MYC2*. Consistent with this, mass spectroscopy of extracts from tubers and stems infected with either *Pba* SCRI1043 and *Pbr* ICMP19477 confirmed the production of both CFA and its amino acid conjugate CFA-valine during infection.

In summary, this thesis presents new information on the defence response deployed by potato to two important SRE pathogens and novel insights into CFA-mediated host susceptibility.

Keywords: Coronafacic acid, *Pectobacterium*, RNA sequencing, *MYC2*, *JAR1*

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Abbreviations

Δ- Deletion
ABA- Abscisic Acid
AU- Auxin
CFA- Coronafacic Acid
CFA-Ile- Coronafacic Acid-Isoleucine
CFA-Val- Coronafacic Acid-Valine
CFL- Coronafacate Ligase
CFU- Colony Forming Unit
CMA- Coronamic Acid
COR- Coronatine
DAMP- Damage-Associated Molecular Pattern
DE- Differentially Expressed
DEG- Differentially Expressed Gene
DNA- Deoxyribonucleic Acid
Dpi- Days Post Inoculation
ET- Ethylene
ETI- Effector-Triggered Immunity
ETS- Effector-Triggered Susceptibility
FDR- False Discovery Rate
FPKM- Fragment Per Kilo base of transcript per Million
GA- Gibberellic Acid
GC-MS- Gas Chromatography-Mass Spectroscopy
GO- Gene Ontology
HAI- Horizontally Acquired Island
Hpi- Hours Post Inoculation
HR- Hypersensitive Reaction
JA- Jasmonic Acid
JA-Ile- Jasmonic Acid-Isoleucine
LAR- Local Acquired Resistance
m/z- Mass-to-charge ratio
MAMP- Microbe-Associated Molecular Patterns
MeJA- Methyl Jasmonate
min- Minute
MM- Minimal Media
NBS-LRR- Nucleotide-Binding Site Leucine-Rich Repeat
NGS- Next Generation Sequencing
NZGL- New Zealand Genomics Limited
OD- Optical Density
PAMP- Pathogen-Associated Molecular Pattern
PCA- Principal Component Analysis
PCWDE- Plant Cell Wall Degrading Enzyme
PGSC- Potato Genome Sequencing Consortium
PRR- Pattern Recognition Receptor
PTI- Pathogen-Triggered Immunity
qRT-PCR- Quantitative Reverse Transcription-Polymerase Chain Reaction
RIN- RNA Integrity Number
RNA- Ribonucleic Acid

RNA-seq- RNA Sequencing
ROS-Reactive Oxygen Species
Rt- Retention Time
SA- Salicylic Acid
SAR- Systemic Acquired Resistance
SDW- Sterile Distilled Water
SRE- Soft Rot *Enterobacteriaceae*
TF- Transcription Factor
TSS- Type Secretion System
VPE- Vapour Phase Extraction

Abbreviation of Genes

12-opr3- 12-Oxophytodienoate reductase 3
ACO- ACC Oxidase
ACS- ACC Synthase
AOC- Allene Oxide Cyclase
AOS- Allene Oxide Synthase
BAK1-Brassinosteroid insensitive 1-Associated Kinase 1
BOS1- Botrytis Susceptible 1
CaM- Calmodulin protein
CBLs- Calcineurin B-like proteins
CDPKs- Ca2+ dependent protein kinase
CHIB- Chitinase B
CML- Calmodulin-like
COI1- Coronatine Insensitive 1
EDS1- Enhanced Disease Susceptibility 1
EFR- EF-TU Receptor
EF-TU- Elongation Factor-Thermo Unstable
EIL- Ethylene Insensitive Like
EIN- Ethylene Insensitive
EPS- Extracellular Polysaccharides
ERFs- Ethylene Response Factors
FLS2- Flagellin Sensing 2
Grx- Glutaredoxin
ICS1- Isochorismate Synthase 1
JAR1- JA amino acid Synthase
JAZ- Jasmonate Zim domain protein
JMT- JA carboxyl Methyl Transferase
LecRks- Lectin Receptor Kinases
Lox- Lipoxygenase
LPS- Lipopolysaccharides
LRR- Leucine-Rich Repeat
MAPK- Mitogen-Activated Protein Kinase
NPR1- Non-Expression of PR1
OG- Oligogalacturonides
ORF59-Octadecanoid-Responsive Arabidopsis AP2/ERF59
PA- Phosphatidic Acid
PAD3- Phytoalexin Deficient 3
PAL- Phenylalanine Ammonia-Lyase
PDF1.2- Plant Defensin 1.2
Pep1&2- Peptides
PEPR1&2- Peptide Receptors 1&2
PGN- Peptidoglycan
PLC &D- Phospholipases C&D
PR- Pathogenesis-Related
Prx- Peroxidase
RBOHs- Respiratory Burst Oxidase Homologs

RLK- Receptor-Like Kinase
RLP- Receptor-Like Protein
SAM- S-adenosylmethionine
Sn- Snakin
Trx- Thioredoxin
VSP2- Vegetative Storage Protein 2
WAK1 &2- Wall-Associated Kinase 1&2

Chapter 1 Introduction

1.1 Potato

Potato (*Solanum tuberosum*), along with other economically important plants such as tobacco (*Nicotiana tabacum*), pepper (*Capsicum annuum*), tomato (*Solanum lucopersicum*) and eggplant (*Solanum melongena*) belongs to the family *Solanaceae*. It is an important dietary source of starch, protein, vitamins and antioxidants (Birch et al., 2012; Potato Genome Sequencing Consortium, 2011), and as a result it is cultivated across a wide geographical range. Indeed, the worldwide importance of potato has grown rapidly, with the production of potato tubers increasing from 327 Million tonnes in 2000 to 376 Million tonnes in 2013 (FAO Statistical Yearbooks - World food and agriculture, 2016) . It is now the third most important staple food source in the world (Wiesel et al., 2015).

Europe, North America, and countries of the former Soviet Union were the major potato producers and consumers of potato until late in the 19th century, when a sharp geographical shift in cultivation lead to production in developing countries (in Asia, Africa and Latin America) overtaking that in developed countries (*Top Potato Producing Countries*, 2016). According to recent statistics, almost one-third of potatoes are now harvested in China or India, with China leading the potato production industry (*FAO Statistical Yearbooks - World food and agriculture*, 2016). Of the total production, approximately 50% of potatoes are estimated to be consumed fresh, 10% are used as seed tubers for the production of the next generation, and the rest are processed or used as animal feed (Birch et al., 2012).

1.2 Potato production in New Zealand

In New Zealand, about 10,000 hectares of land are used to cultivate potato, with an estimated production of 525,000 metric tonnes of potato tubers every year (Zook & Kuć, 1991). Approximately 56% of the area grown is destined for processed products while 33% and 11%

are used for table and seed production, respectively. Pukekohe, Manawatū, South Canterbury and Waikato are the primary areas where potatoes are grown commercially. Overall, New Zealand potato production is worth approximately $800 Million and total potato exports are worth $100 Million (*Fresh Facts - New Zealand Horticulture*).

1.3 The impacts of disease on potato production

Approximately 22% of the global yield of potato is lost due to pest or viral, bacterial or fungal diseases (*FAO Statistical Yearbooks - World food and agriculture*, 2016). As potatoes are vegetatively propagated, the main sources of inoculum for many pathogens are infected seed tubers or contaminated parent plants (Czajkowski et al., 2015; Czajkowski et al., 2011; De Boer, 2002). As infected seed tubers or contaminated parent plants are often destined for national distribution or even global markets, many countries have established seed certification systems or import health standards to reduce the risks associated with such inoculum sources (Frost et al., 2013). The spread of pests and pathogens, along with the diseases they cause, continues to be a major constraint to production globally due to the intensification of farming, the increased global movement of food and the lack of formal seed certification or import standards in the developing world.

1.4 Soft rot and blackleg of Potato

Tuber soft rot and blackleg are diseases of worldwide economic significance to the potato industry. Blackleg alone is considered to be of primary importance in seed production alongside bacterial wilt disease caused by *Ralstonia solanacearum* and ahead of ring rot and common scab caused by *Clavibacter michiganenesis* subsp. *sepedonicus* and *Streptomyces scabies*, respectively (Czajkowski et al., 2011).

Tuber soft rot is associated with seed piece decay or with soft rotting of progeny tubers in the field or during storage (Perombelon, 1992, 2002). Tuber soft rots are characterised by wet

grainy white/brown rots, which have black margins separating the rotting flesh from the healthy tissue (De Boer, 2004). Initial symptoms of soft rot infection are quite diverse. For example, early seed piece decay can result in infection of the sprout or in non-emergence (Perombelon & Kelman, 1980). In contrast, an important source of soft rot infection in progeny tubers is through the stolon, and the potato tubers attached to a plant with infection often develop soft rot symptoms at the stolon attachment site (Perombelon, 2002; Perombelon & Hyman, 1989). An alternate source of soft rot infection in progeny tubers is contaminated soil and water. Rotting of the seed tuber (mother tuber) and the below ground stem leads to the release of the pathogen into the soil water, which is then distributed throughout the root zone in which the progeny tubers grow (Perombelon, 2002; Perombelon & Hyman, 1989). The pathogen subsequently enters the lenticel of the progeny tubers or through wounds and either becomes latent or initiates tissue maceration, depending on the environmental conditions at the time of infection (Charkowski, 2006). Soft rot infection during post-harvest storage spreads amongst adjoining tubers when the liquid from a rotting tuber seeps into other tubers (Czajkowski et al., 2015; Czajkowski et al., 2011).

Blackleg disease is characterised by the rotting or wilting of stems of growing plants in the field (Perombelon, 1992, 2002). Symptoms are expressed when the pathogen predominates in the rotting mother tuber, invades the stem and multiplies in the xylem vessels (Lumb et al., 1986; Perombelon, 2002). This occurs primarily under wet conditions, resulting in the stems of diseased plants showing black to light brown decays that extend from three to 30 centimetre from the soil surface. Infection of stems can still occur under dry conditions, with leaves of infected mature plants rolling upwards, becoming yellow, wilting, and often dying (Charkowski, 2006; Perombelon & Kelman, 1980). The pith region in the stem is often particularly susceptible to decay, and disease of this tissue may extend upward far beyond the visible external disease lesion. Blackleg often develops soon after plant emergence. Such young, infected plants usually fail to develop and typically die (Perombelon & Kelman, 1980). The disease cycle for tuber soft rot and blackleg are described in Figure 1.1.

Figure 1.1: Disease cycle of bacterial soft rot in potato (De Boer, 2004).

1.5 The Soft Rot *Enterobacteriaceae*

Tuber soft rot and blackleg of potatoes are caused primarily by bacteria belonging to the family *Enterobacteriaceae*. Accordingly, these bacteria are collectively known as the soft rot *Enterobacteriaceae* (SRE) (formally the Soft Rot Erwinias). The *Enterobacteriaceae* family (Charkowski, 2006; Czajkowski et al., 2011) includes both plant and animal pathogens such as *Erwinia amylovora*, *Escherichia coli*, *Salmonella* spp., and *Yersinia* spp. SREs are pectinolytic, gram-negative, rod-shaped, facultative anaerobes that cause diseases on a variety of crops (Perombelon & Kelman, 1980).

The SRE were formally classified as *Erwinia carotovora*, *E. cacticida* or *E. chrysanthemi* (Young et al., 1978; Young et al., 1996). Based on pathogenicity and host specificity, *E. carotovora* (Winslow et al., 1920) was subdivided into five subspecies: *E. c.* subsp*. atrosepticum, E. c.* subsp*. betavasculorum*, *E. c.* subsp. c*arotovora*, *E. c.* subsp. o*dorifera*, and *E. c.* subsp.

wasabiae. The classification of these bacteria was subsequently revised, with the SRE falling either into the genus *Pectobacterium* or *Dickeya*. The genus *Pectobacterium* was first proposed by Waldee (1945), but it was the revision based on 16s rDNA sequence and their ability to grow at 39°C that led to the placement of the soft rotting *'carotovora'* species in the genus *Pectobacterium* and *E. chrysanthemi* in *Dickeya* (Hauben et al., 1998; Laurila et al., 2008; Samson et al., 2005). DNA–DNA hybridization, phenotypic characterisation and serological testing subsequently led to the elevation of four *Pectobacterium* subspecies to species level. As a result, these four taxa are now known as *Pectobacterium atrosepticum* (*Pba*), *Pectobacterium carotovorum* subsp *carotovorum* (*Pcc*), *Pectobacterium betavasculorum,* and *Pectobacterium wasabiae* (*Pw*) (synonyms; *Erwinia carotovora* subsp. a*troseptica, Erwinia carotovora* subsp. c*arotovora, Erwinia carotovora* subsp. *betavasculorum,* and *Erwinia carotovora* subsp. *wasabiae*, respectively) (Gardan et al., 2003). A fifth taxon, *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbr*), was later detected in Brazil, which was responsible for blackleg disease on potato plants (Duarte et al., 2004; van der Merwe et al., 2010). This pathogen was subsequently identified in potato growing regions across the globe, including South Africa (van der Merwe et al., 2010), Canada (De Boer et al., 2012), New Zealand (Panda et al., 2012), Syria (Nabhan et al., 2012), Korea (Lee et al., 2014) and more recently the Netherlands (Leite et al., 2014).

Pba has a narrow host range and is restricted mainly to potato plants in temperate regions, with maximal disease symptoms occurring at below 25°C (Perombelon, 2002; Perombelon & Kelman, 1980). The inability of *Pba* to infect other host plants could be due to host specificity or ecological factors favouring its survival in vegetatively reproduced crops from one season to the next. Certainly, *Pba* is considered a seed tuber borne pathogen and does not appear to survive in the soil for longer than a year unless contained in diseased tubers (Czajkowski et al., 2011). In contrast, *Pcc* and *Pbr* have the broadest host range affecting crops in temperate and subtropical regions, respectively. They multiply and persist in the root zones of host and nonhost crop and weed species, remaining latent in seed tubers unless the environmental conditions are favourable for causing disease (Ma et al., 2007; Marquez-Villavicencio et al., 2011; Perombelon, 2002; Toth et al., 2003).

Dickeya spp. are pathogenic to many plants, usually affecting potatoes grown in tropical or sub-tropical climates. However, they have also been identified in temperate parts of Europe (Toth et al., 2011). Seven species are currently recognised in *Dickeya; D. dadantii*, *D. dianthicola*, *D. zeae*, *D. paradisiaca*, *D. chrysanthemi*, *D. aquatica, D. solani* (Samson et al., 2005). *D. dadantii* is divided into two subspecies: *D. dadantii* subsp. *dadantii* and *D. dadantii* subsp. *dieffenbachiae* (Brady et al., 2012). When compared to pectobacteria, *Dickeya* spp. have a broad host range (Ma et al., 2007). Furthermore, with the exception of *D. aquatica*, all the species and subspecies of *Dickeya* have been isolated from potatoes (Van Der Wolf et al., 2014). *D. dianthicola* and *D. solani* have been identified as major potato pathogens in Europe (Toth et al., 2011). *Dickeya* spp. can initiate disease symptoms from a lower inoculum and are capable of spreading through plant vascular tissues (Czajkowski et al., 2010). Furthermore, several *Dickeya* spp. are considered to be highly aggressive pathogens, despite requiring higher optimal temperatures for disease development (Janse & Ruissen, 1988; Toth et al., 2011; Wolf et al., 2007).

1.6 The changing dynamics of Soft Rot *Enterobacteriaceae* on potato

Historically, it was considered that *Pba* was the primary organism responsible for blackleg disease of potato plants during the growing season (Perombelon, 2002; Perombelon & Kelman, 1980; Sledz et al., 2000), though all SREs were capable of causing soft rot. However, in the last decade, this restricted view of SRE pathogenesis was challenged, when improved diagnostics and systems for taxonomic classification revealed that the list of SRE pathogens believed to cause blackleg was more extensive than previously thought and that the populations of SRE on potato were more dynamic than predicted. For example, *Pw* (Moleleki et al., 2013; Pitman et al., 2010) and *Pbr* (Duarte et al., 2004; Leite et al., 2014; Panda et al., 2012; van der Merwe et al., 2010) were found to elicit blackleg, the former after infiltration into tubers during experimental field trials (De Boer et al., 2012). *D. solani* also emerged as a threat to potato production in Europe (Toth et al., 2011). The emergence of such variants or improvements in their detection resulted in what seem to be almost annual fluctuations in the populations of SRE causing blackleg in potato crops. Certainly, the Dutch reported that in

2015 *Pbr* emerged as the predominant SRE in seed tubers, after previous outbreaks of *Pw* and *D. solani* in 2014 and 2103 (*New bacteria main cause of blackleg in Dutch seed potatoes*, 2016). In the US, new reports have suggested *D. dianthicola* is emerging as the predominant SRE in seed crops after years of being considered a relatively benign member of the SRE (*Late season management of bacterial disease*, 2015). The reasons for the recent emergence of new SREs or more aggressive forms of existing species remain unknown, but it is speculated that it may be related to differences in the repertoire of pathogenicity factors they have acquired during their evolution.

1.7 Pathogenicity determinants in *Pectobacterium* infecting potato

Pectobacterium spp. infecting potato are characterised by the production of plant cell wall degrading enzymes (PCWDEs) that result in the maceration of host tissues. Genomics research, however, has revealed the presence of a plethora of additional virulence determinants that are involved in colonisation of the host (Bell et al., 2004; Glasner et al., 2008; Koskinen et al., 2012; Panda et al., 2015). The variety of these determinants, which include flagella proteins, motility lipopolysaccharides, exopolysaccharides, iron acquisition, secretion systems (including type III, type IV and type VI secretion systems) and phytotoxins (Bell et al., 2004; Glasner et al., 2008; Koskinen et al., 2012; Panda, 2014; Toth et al., 2006), suggests that *Pectobacterium* spp. coordinate the production of an array of different virulence factors that promote infection at different stages in the interaction with the host. These infection stages include the colonisation of the pathogen, population maintenance prior to the onset of the infection, and necrosis of the plant tissues during the later stages. For instance, *Pbr* has been shown to enter the plant in a motile phase, but to convert to a sessile life stage once it enters the stem and reaches high cell densities (Moleleki et al., 2016). Due to their relevance to this thesis, only PCWDEs, the secretion systems and the putative phytotoxin coronafacic acid (CFA) are discussed in detail.

1.7.1 Plant cell wall degrading enzymes

The capacity of *Pectobacterium* spp. to infect plants relies largely on the production of a wide range of PCWDEs, so it is not surprising that genome sequencing has identified at least 20 putative PCWDEs in *Pba* SCRI1043 and 64 in *Pbr* ICMP19477 (Bell et al., 2004; Glasner et al., 2008; Panda, 2014). The genome of *Pw* SCC3193 is also predicted to encode 39 putative pectinases, cellulases and proteinases, most of which have been previously reported in other sequenced *Pectobacterium* strains (Koskinen et al., 2012; Nykyri et al., 2012). Of all the PCWDEs identified in *Pectobacterium* spp., pectinases are believed to be the most important exoenzymes. They break down the pectin in the middle lamella of plant cell walls causing tissue and cell damage leading to cell leakage. Many pectinases such as pectate lyase (Pel), pectin lyase (Pnl), pectin methyl esterase (Pme) and polygalacturonases (Peh) have been implicated in pathogenesis and have been identified in multiple forms (isoenzymes) (Perombelon, 2002; Toth et al., 2003). Comparative genomics of *Pba, Pcc* and *Pbr* has revealed that all three species encode orthologous PCWDEs including ten Pel, one Pnl, four Peh, two cellulases and one rhamnogalacturonase (Glasner et al., 2008). Furthermore, both *Pcc* and *Pbr* encode other putative PCWDEs that are not present in *Pba* (Glasner et al., 2008; Panda, 2014). *Pbr* ICMP19477 also encodes an additional putative rhamnogalactoronate lyase, and a novel M20 family peptidase Pepv (Panda, 2014), which may contribute to its greater aggressiveness than other strains.

1.7.2 Secretion systems

In gram-negative bacteria, secretion systems are involved in the transport of numerous proteins into the host, including those associated with the virulence. Plant pathogenic bacteria encode several secretion systems including the Type 1 secretion system (T1SS), T2SS, T3SS, T4SS, T5SS, T6SS and T7SS. In *Pectobacterium* spp. the T1SS and T2SS are involved in delivering the PCWDEs and other virulence factors proximal to the host cells. Studies in *Pba* SCRI1043 and *Pba* SCC3193 have shown that the T1SS-secreted proteases and other virulence

factors are required for pathogenicity in potato (Marits et al., 1999; Perez-Mendoza et al., 2011). In addition, T2SS mutant *Pectobacterium* strains are impaired in the secretion of pectic enzymes and cellulose, which renders them avirulent (Pirhonen et al., 1991; Reeves et al., 1993).

The T3SS is capable of secreting proteins into the extracellular space and translocating proteins into the host cell. The *hrp* genes encode the T3SS apparatus that mediates the transfer of these 'effector' proteins into the eukaryotic host cell. The T3SS is often required for a pathogen to grow and to cause disease on susceptible plants, but it can elicit defence responses in resistant plants (Galan & Collmer, 1999; Greenberg & Vinatzer, 2003). Comparative genomics has identified that, except for HrpK (T3SS protein), the T3SS is conserved across *Pba*, *Pcc* and *Pbr* (Glasner et al., 2008). *Pba* SCRI1039 mutants with Tn*5* insertions in *hrcC*, *hrcV*, *hrpN* and *dspE/A* were tested for their virulence on potato stems relative to the wild-type strain. Disease lesions were reduced considerably in plants inoculated with the mutant strains, suggesting that the T3SS and effector DspE/A are involved in virulence of this pathogen on potato (Holeva et al., 2004b). In addition to the main secretory component of the T3SS, *Pectobacterium* spp. also encode T3SS effector proteins resembling the *srfABC* T3SS-associated gene cluster of *Salmonella* species (Lostroh & Lee, 2001). Orthologs of *srfc*, secreted through T3SS, were also identified in the plant pathogenic bacterium *Pseudomonas syringae* (Petnicki-Ocwieja et al., 2002).

The T4SS is required for plasmid conjugation and is also capable of secreting proteins and nucleic acids to the host cell. Unlike T3SSs, T4SSs are present in different locations in the genome of *Pba*, *Pbr* and *Pcc*. In *Pcc,* only a remnant of the T4SS is present, however, in *Pba* the T4SS is involved in the virulence of the pathogen on potato (Bell et al., 2004). In *Pba* SCRI1043, the T4SS includes genes *virB1* to *virB11*, with a mutation in *virB4* leading to a significant reduction in blackleg on potato (Bell et al., 2004).

The T5SS includes an autotransporter and a two partner secretion system (*HecAB*) (Henderson et al., 2004). Mutation in the *hecA* gene in *Dickeya* impairs attachment, aggression and subsequent death of epidermal cells of tobacco seedlings, suggesting a role in the pathogenicity of the pathogen (Rojas et al., 2004). However, the precise role of *HecAB* in the virulence of *Pectobacterium* spp. is yet to be confirmed.

T6SS encodes a cluster of 15-20 genes. Hcp (Hemolysin coagulated-like protein) and VgrG (Valine glycine rich) proteins are the secreted substrates of this system. Genome sequencing of *Pectobacterium* spp. has confirmed the presence of the *hcp* genes (Bell et al., 2004; Panda, 2014). *In vitro* studies have also shown that addition of plant extract (stem extract and the tuber extract) results in the induction of the T6-secreted VgrG and Hcp proteins (Mattinen et al., 2007). Furthermore, mutations in the T6SS of *Pba* SCRI1043 resulted in a significant reduction in disease symptoms in potato tubers and stems (Liu et al., 2008). In *Pectobacterium* spp. and *Dickeya* spp., the T6SS is related to *in planta* survival and virulence of these pathogens (Liu et al., 2008; Nykyri et al., 2012).

1.7.3 Phytotoxins

Genome sequencing of *Pba* SCRI1043 and *Pbr* ICMP19477 has identified two biosynthetic clusters in each of these SRE that encode products similar to known phytotoxins (Bell et al., 2004; Panda et al., 2015). The first cluster in each strain has two putative non-ribosomal peptide (NRP) synthase genes. Together, these synthetases appear to encode an NRP. The NRPs produced by the two strains are not only related to one another, but have similarity to syringomycin, a phytotoxin in *P. syringae* (Bell et al., 2004; Panda, 2014). Syringomycin induces necrosis in plant tissues, causing expansion of the pores in the plasma membrane that leads to nutrient and ion leakage and consequently to cytolysis (Bender et al., 1999). Despite the purported similarities to syringomycin, the function of these NRPs has not been investigated in either *Pba* SCRI1043 or *Pbr* ICMP19477.

The second putative phytotoxin biosynthetic cluster in both *Pba* SCRI1043 and *Pbr* ICMP19477 includes nine genes (*cfa1-cfa8B*) with similarity to those genes encoding enzymes involved in the biosynthesis of coronafacic acid (CFA) in several taxa of *P. syringae* (Figure 1.2) (Bell et al., 2004; Panda et al., 2016). CFA, a molecular mimic of methyl jasmonate (MeJA), is a component of coronatine (COR), an important phytotoxin in several taxa of *P. syringae.* In *P. syringae*, COR is produced by ligation of CFA to coronamic acid (CMA), an ethylcyclopropyl amino acid (Bender et al., 1999). Unlike *P. syringae*, *Pba* SCRI1043 and *Pbr* ICMP19477 lack the *cma* genes, which suggests COR is not produced by these bacteria. They do, however, encode a gene for the synthesis of coronafacic ligase (CFL), which is used to conjugate CFA to CMA to form COR (Bender et al., 1993). Thus, it is hypothesised that in SRE, CFA is conjugated to another molecule to form a conjugate similar, but distinct from COR (Bell et al., 2004; Panda et al., 2016).

1.7.3.1 CFA and its role in pathogenicity of SRE

Inactivation of CFA production in *Pba* SCRI1043 results in reduced virulence on potato stems (Bell et al., 2004). The functional target of CFA remains unknown but, given prior work on COR in *P. syringae,* it is predicted to be involved in manipulating hormone signalling during the host response to the pathogen (Geng et al., 2014; Sheard et al., 2010; Uppalapati et al., 2005; Uppalapati et al., 2007; Zheng et al., 2012). In *P. syringae*, COR is structurally related to the plant hormone Jasmonic acid-Isoleucine (JA-Ile) (Katsir et al., 2008). It functions as a molecular mimic of this plant hormone by promoting COI1-JAZ interactions that activate JA signalling and thereby suppress SA-mediated plant defence (Katsir et al., 2008; Melotto et al., 2008; Zheng et al., 2012). Salicylic acid (SA) mediated plant defence is usually initiated in response to biotrophic/hemibiotrophic pathogens such as *P. syringae* (Glazebrook, 2005; Glazebrook et al., 1996; Nawrath & Metraux, 1999; Rogers & Ausubel, 1997; Zhou et al., 1998). These data suggest COR manipulates plant hormone signalling to enhance the susceptibility of the host to infection.

To assess whether COR and/or CFA functioned as jasmonate analogues, expression of several jasmonate responsive genes was monitored in tomato leaves inoculated with purified COR, CFA

or CMA using microarrays. This analysis revealed that the jasmonate responsive genes, including *lox2* (which encodes for Lipoxygenase and is induced on treatment with JA or wounding) and *PDF 1.2*, were strongly induced 12 hours post inoculation (hpi) with COR. These data suggested that COR acts as a jasmonate analogue and plays a vital role in expression of disease in *P. syringae*. Although there was a significant overlap (~39%) in the COR and CFA-mediated host response, It was COR that induced the maximum number of JA-related responses (Uppalapati et al., 2005).

Genome sequencing of *Streptomyces scabies* 87-22 identified a region similar to the CFA cluster in *Pba* SCRI1043 and *P. syringae* (Bignell et al., 2010). *Streptomyces* spp. cause scab on economically valuable root and tuber crops including potato (Loria et al., 2006). In *S. scabies*, the CFA cluster is encoded by eight genes (*cfa1-cfa8*) and is expressed under conditions that induce thaxtomin A production, the key virulence factor in *S. scabies* (King & Calhoun, 2009). The CFA mutants of *S. scabies* show reduced virulence on potato, but still cause extensive stunting of the roots and shoots as well as chlorosis and eventual death of the host plant (Bignell et al., 2010). As in *Pba* SCRI1043, CMA is not produced by *S. scabies*, suggesting that the CFA-like cluster contributes directly to the aggressiveness of *S. scabies* on potato, even in the absence of CMA. Recent chromatography studies in *S. scabies* have reported the production of CFA and its coronafacoyl conjugate CFA-Isoleucine (CFA-Ile) (Fyans et al., 2015). Furthermore, pathogenicity assays using culture supernatants from *S. scabies* induced potato hypertrophy, in a similar manner to purified COR (Fyans et al., 2015). Thus, in the absence of CMA, CFA appears to form conjugates with other amino acids to create the active derivative of CFA.

The CFA biosynthetic clusters in *Streptomyces* (Bignell et al., 2010), *P. syringae* (Bender et al., 1989) and *Pectobacterium* (Bell et al., 2004) appear to have been acquired by horizontal gene transfer, as they are harboured on mobile genetic elements in each genus. In *Pba* SCRI1043, for instance, the *cfa* gene cluster is present on a horizontally acquired island (HAI) known as HAI2 (Bell et al., 2004). As expected, deletion of this island from the genome of *Pba* SCRI1043 using CRISPR-based genome editing resulted in reduced virulence in stem assays (Panda et al., 2016). PCR and comparative genome analysis of multiple *Pba* strains has confirmed the presence of HAI2 or HAI2-like islands in all strains tested, suggesting that although CFA and

HAI2 only enhance virulence, there must be strong selective pressure for both to be retained in the bacterial population (Panda et al., 2016).

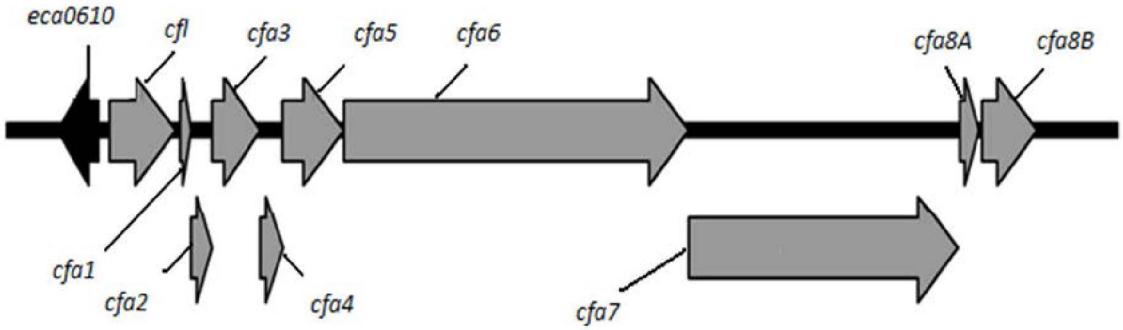


Figure 1.2: Organisation of the coronafacic acid biosynthetic cluster in *Pectobacterium atrosepticum* SCRI1043.

1.8 Plant response to infection by pathogens

The presence of virulence genes like phytotoxins on mobile genetic elements indicates that although the virulence genes are not essential to the bacterial host, they must confer considerable advantage under certain conditions. This can be explained by the arms race that exists between pathogens and their host, in which a pathogen evolves to infect a susceptible host. In response, the plant develops mechanisms to combat these attacks. Under renewed pressure, the pathogen rapidly develops new ways to invade its host, find an alternative host or change its lifestyle altogether. A seemingly effective way to do this is to acquire entire biosynthetic clusters by virtue of horizontal transfer.

A plant's attempts to restrict pathogen attack are known as a defence response. Plants have evolved an array of defence responses to combat attack by pathogens. Plants, however, lack an adaptive immune system, which means these defence responses are mediated primarily by the innate immunity of each cell and systemic signals that are emitted from the site of infection to trigger this immunity (Dye, 1981; Kwon et al., 1997). Plants also trigger a range of physiological changes in response to infection. These include the induction of reactive oxygen species (ROS) and cell wall strengthening (callous deposition) at the site of infection,

hormonal regulation, synthesis of secondary metabolites and antimicrobial products such as phytoalexins. The coordinate effect of these physiological changes results in the ability of the plant to protect itself from pathogen attack (Cohn et al., 2001; Jones & Dangl, 2006).

Two branches of plant defence in response to microbial attack have been recognised (Dodds & Rathjen, 2010; Jones & Dangl, 2006). The first involves transmembrane pattern recognition receptors (PRRs) that respond to pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular patterns (MAMPs) (Boller & Felix, 2009). The second system uses the polymorphic nucleotide binding site-leucine rich repeat (NBS-LRR) protein products that are encoded by *R* genes (Dangl & Jones, 2001). Jones and Dangl (2006) presented a 'zigzag' model for the evolution of plant immunity (Figure1.3), in which the response to microbial pathogens was divided into four phases. Phase 1 of this model involves pattern-triggered immunity (PTI), which is produced when PRRs recognise PAMPs or damage-associated molecular patterns (DAMPs) such as flagellin, chitin, glycoproteins and lipopolysaccharides or plant signals associated with cell damage. In phase 2, a successful pathogen enters the host and deploys effectors which interfere with the PTI resulting in effector-triggered susceptibility (ETS). In phase 3, if an effector is recognised specifically by one of the NBS-LRR it results in effector–triggered immunity (ETI). ETI is represented as an amplified PTI response which results in a hypersensitive response (HR). In the final phase, the pathogen suppresses the ETI response either by discarding the specific effector or by obtaining or activating a new effector (Jones & Dangl, 2006). Activation of PTI and ETI results in elicitation of defence-associated signalling and crosstalk between the pathways involved in signalling.

Figure 1.3: Schematic representation of the "Zig-Zag" model in plant immunity (Jones & Dangl, 2006). Note: PTI, ETI and ETS represents pattern-triggered immunity, effector-triggered immunity and effector-triggered susceptibility.

Plants also possess 'acquired resistance', otherwise known as systemic acquired resistance (SAR). Acquired resistance develops in plants following primary pathogen attack, when the surrounding or the remote tissues become resistant to subsequent infections. SAR was first detected in the early 1960's in tobacco plants, where increased resistance was detected in distal tissues of plants previously challenged with tobacco mosaic virus (TMV) (Ross, 1961). SAR confers long-lasting protection against a broad spectrum of infections in plants and is characterised by the induced expression of several pathogenicity related genes (*PR* genes) and occurs both locally and systemically. SAR involves the rapid accumulation of SA occurring initially in the infected tissue, which then triggers a more systemic dispersal of SA (Gao et al., 2015; Malamy et al., 1990). Recent studies have shown the importance of SA in both the local defence response and in the establishment of SAR (Durrant & Dong, 2004; Gao et al., 2015).

1.8.1 Pattern-triggered Immunity

PTI in plants forms the first layer of the innate immune response and is based on the sensitive perception of PAMPs or DAMPs though PRRs at the plant cell surface (Dodds & Rathjen, 2010; Jones & Dangl, 2006). PAMPs, a structural component of the microbial cell are highly conserved within microbes and are essential for pathogen survival and fitness within the host cell (Nicaise et al., 2009). In contrast, DAMPs, are host-derived signals. Secretion of PCWDEs by pectolytic pathogens results in cell wall degradation and release of oligopeptides; these oligopeptides act as endogenous elicitors or DAMPs. DAMPs typically appear in the apoplast, and like PAMPs result in the activation of PTI (Boller & Felix, 2009; Dodds & Rathjen, 2010; Matzinger, 2002). Surface localised PRRs in plant are either receptor like kinase (RLKs) or receptor like proteins (RLPs) and contain ectodomains including leucine rich repeats, lysine motifs, lectin motifs or epidermal growth factors (EGF)-like domains (Dodds & Rathjen, 2010). LRR type PRRs have been associated with the binding of proteins or peptides like bacterial flagellin, EF-Tu, or Pep peptides (Chinchilla et al., 2006; Sun et al., 2013; Zipfel et al., 2006), the PRRs containing other domains are involved in the recognition of carbohydrate containing molecules including the fungal chitin, bacterial peptidoglycans, extracellular ATP or plant cell wall derived oligogalacturonides (Brutus et al., 2010; Choi et al., 2014; Willmann et al., 2011). Downstream signalling relating to PTI includes early defence responses such as ion flux, oxidative burst, stomatal closure, callose deposition and hormone-mediated defence regulation (Nicaise et al., 2009; Zipfel, 2008).

1.8.1.1 PAMP-triggered Immunity

PAMPs including bacterial flagellin (*flg22*) (Gómez-Gómez & Boller, 2000), elongation factor Tu (EF-Tu) (Kunze et al., 2004) and Peptidoglycan (PGN) (Liu, Liu, et al., 2012; Willmann et al., 2011) result in the activation of PTI. Perception of PAMPs by the PRRs results in the activation of signalling events, commonly known as the general defence response in plants. Perception of PAMPs by plant cells also results in SAR (Mishina & Zeier, 2007). One of the well-characterised PRRs is the leucine-rich repeat-receptor kinase (LRR-RK) flagellin sensing 2

(FLS2), which is involved in sensing bacterial flagellin or the derivative of the conserved flg22 epitome present in the N-terminus (Boller & He, 2009; Zipfel, 2009). LRR-RK FLS2 belongs to the subfamily XII of LRR-RKs. Homologs of FLS2 have been identified in *Arabidopsis* (Gómez-Gómez & Boller, 2000), tomato (Robatzek et al., 2007), rice (Takai et al., 2008) and tobacco (Hann & Rathjen, 2007). Studies in *Arabidopsis* have confirmed the role of FLS2 in bacterial resistance; pre-treatment of *Arabidopsis* plants with the *flg22* results in the reduced growth of *P. syringae*, while *fls22* mutants are susceptible (Zipfel et al., 2004). Furthermore, lack of flagellin recognition in *Arabidopsis* results in enhanced susceptibility to a wide range of non-adapted bacterial pathogens (De Torres et al., 2006; Li et al., 2005; Zipfel, 2009). Bacterial EF-Tu is another well-characterised PRR in *Brassicaceae* family and have since then been identified in *Arabidopsis* (Kunze et al., 2004; Nicaise et al., 2009; Zipfel et al., 2006). Though efl18 (EF-Tu) is secreted intracellular, the release of EF-TU by dying bacteria results in recognition by the plant EF-TU receptor (EFR). Like FLS2, EFR belongs to the subfamily XII (Zipfel et al., 2006). *Arabidopsis efr* mutants are susceptible to *P. syringae* and more amenable to transformation by *Agrobacterium tumefaciens* (Nicaise et al., 2009; Zipfel, 2009). Furthermore, transient heterologous expression of *AtEFR* in tobacco, a plant lacking EFR, results in the elf18 perception and activation of defence response (Nicaise et al., 2009).

In addition to FLS2 and EFR, LYM1 and LYM3 orthologs of chitin binding protein (CEBiP) have been shown to bind to PGN, the major component of bacterial cell wall (Willmann et al., 2011). In *Arabidopsis*, the perception of PGN by LYM1 and LYM3 required the CERK1 (lysine motif containing domain required for chitin perception in *Arabidopsis*), however, the precise mechanism involved in the PGN perception in *Arabidopsis* is yet to be understood (Macho & Zipfel, 2014; Willmann et al., 2011). Although various other bacterial PAMPs have been suggested, their corresponding PRRs are yet to be discovered; FLS2 and EFR are the only known and fully characterised bacterial PRRs (Macho & Zipfel, 2014; Zipfel, 2009).

1.8.1.2 DAMP-triggered immunity

In addition to PAMPs, plants also sense endogenous damage caused by wounding, chewing insects or herbivores or as a result of PCWDE activity (Boller & Felix, 2009). Necrotrophic/hemibiotrophic pathogens, like *Pectobacterium*, secrete lytic PCWDEs, resulting in the degradation of the plant cell wall. The products produced by the action of these enzymes such as oligogalacturonides (OGs) serve as a source of DAMPs (Brutus et al., 2010; Davidsson et al., 2013; Galletti et al., 2009). The generation and recognition of DAMP signals to induce PTI-mediated defence response varies with the pathogen and depends on the virulence factors it produces (Boller & Felix, 2009). Polygalacturonases produced by necrotrophic pathogens during the early stage of host infection target the homogalacturonan component of pectin, resulting in the release of OGs. In *Arabidopsis*, the perception of OGs is mediated by the wall-associated kinases (WAK). *In vitro* studies have confirmed the ability of WAK1 and WAK2 to recognise pectin (Kohorn et al., 2009). Furthermore, WAK1 binds specifically to OGs (Decreux & Messiaen, 2005). Also, transcriptome studies have confirmed the up regulation of WAK1 in response to OGs, supporting the role of WAK1 in OG-mediated signalling (Denoux et al., 2008). Overexpression of WAK1 also results in induced resistance against the necrotrophic pathogen *Botrytis cinerea* (Brutus et al., 2010). The results of these studies suggest that WAKs could be the PRRs involved in the perception of OGs. Perception of OGs induces PTI response including the cytoplasmic calcium flux, production of ROS, induction of peroxidases, production of chitinase, β-1-3-glucanase, protease inhibitor expression as well as lignin biosynthesis (Davidsson et al., 2013).

In addition to OGs, other DAMPs including small peptides Pep1 and Pep2 have also been identified as DAMPs and have been shown to induce defence response against necrotrophic pathogens (Huffaker et al., 2006; Liu et al., 2013; Ma et al., 2012; Postel et al., 2010; Zipfel, 2013). In *Arabidopsis*, PEPR1 and PEPR2 were identified as the receptors for Pep1 and Pep2 respectively (Huffaker et al., 2006). Constitutive expression of Pep1 and Pep2 results in enhanced resistance to the necrotrophic oomycete *Puthium irregular* (Postel et al., 2010). Recently, lectin receptor kinases (LecRks) have been identified as potential components of PRR complex (Singh & Zimmerli, 2013). For example, LecRK-1.9 was involved in the

recognition of extracellular ATP, which is considered to be released upon cell damage (Choi et al., 2014). Thus, OGs, small peptides and cellular ATP may have a conserved function as the endogenous elicitors of PTI to necrotrophic bacterial, fungal and oomycete pathogens.

1.8.2 Effector-triggered Immunity

ETI forms the second layer of the immune response, triggered when successful pathogens suppress PTI-mediated defence responses by delivering effectors and other virulence factors into the plant cell. Effectors secreted by phytopathogenic bacteria contribute to bacterial virulence by either mimicking or inhibiting cellular functions. Furthermore, effectors have a molecular and enzymatic activity that specifically enables effectors to modify the host target and avoid their intracellular recognition by ETI receptors (Dodds & Rathjen, 2010). Taking into account their ability to avoid recognition, the capacity of a single phytopathogenic bacterium to deliver as many as 15-30 effectors into a plant cell via the T3SS (Dodds & Rathjen, 2010; Jones & Dangl, 2006), and the high degree of variation amongst the bacterial effectors, it seems incredible that many hosts have developed ETI to combat pathogen attack.

Secreted effectors are recognised by specific disease resistance (*R*) genes; *R* genes encode polymorphic NBS-LRR domains (Jones & Dangl, 2006). Based on the N-terminal domain, plant NBS-LRRs are broadly classified into two; the coiled-coil (CC) domain or Toll and interleukin-1-like receptor (TIR) domain NBS-LRRs (Bonardi et al., 2012; Tsuda & Somssich, 2015). Approximately 125 *R* genes have been identified in the *Arabidopsis* COl-0 genome (Jones & Dangl, 2006). Recognition of the effector by the corresponding NBS-LRR triggers ETI-induced defence resistance, often leading to HR-mediated cell death at the infection site. Accordingly, the activation of ETI-mediated defence responses is effective against biotrophic and hemi-biotrophic pathogens, but less effective against necrotrophic pathogens.

Some early cellular events associated with ETI-mediated defence include a rapid influx of calcium ions from the external store, oxidative burst, callose deposition, localised cell death, and hormone-mediated defence signalling. Hence, downstream activation of defence

signalling by PTI and ETI are largely similar, but the two responses differ quantitatively (Boller & Felix, 2009; Dodds & Rathjen, 2010; Jones & Dangl, 2006). A successful ETI-mediated defence response results in the programmed cell death in the form of HR at the site of infection, limiting the spread of the pathogen (Jones & Dangl, 2006). HR induced at the site of infection results in the local acquired resistance (LAR) and SAR at the site of infection and the distal tissues respectively (Sanabria et al., 2010). While SA has been demonstrated to play a vital role in SAR (Gao et al., 2015), SA-mediated signalling has also been linked to PAMPs and DAMPs induced PTI response (Halim et al., 2009).

1.9 Downstream activation of defence responses

Plant perception of PAMPs results in rapid changes in the level, composition and structure of different RNA molecules, proteins and metabolites that precede the defence signalling events. In this thesis, early signalling events relating to both PTI and ETI-mediated defence responses including calcium influx, ROS, hormonal signalling and downstream activation of transcriptional factors and secondary metabolites are discussed in detail.

1.9.1 Calcium-mediated defence

Some of the earliest physiological changes observed after pathogen recognition are ion fluxes across the plasma membrane, including an influx of calcium (Ca^{2+}) in the cytosol (Cheval et al., 2013; Lecourieux et al., 2006). Thus, an increase in the free Ca^{2+} level in cell compartments acts as a signal to activate downstream defence responses. The distinct influx in cytosolic Ca^{2+}, has been observed in *Arabidopsis* leaves treated with various strains of *P. syringae* (Grant et al., 2000). Similarly, plant treatment with PAMPs, including flg22, elf18 and other elicitors of plant defence responses results in cytosolic Ca^{2+} influx (Ranf et al., 2011). The increase in the stimulus-specific Ca^{2+}, also known as Ca^{2+} signature, is recognised by Ca^{2+} binding proteins. Calmodulin (CaM) and Calmodulin-like proteins (CMLs), Ca^{2+} dependent protein kinase (CDPKs), and calcineurin B-like proteins (CBLs) are the three main subfamilies that function as

Ca^{2+} sensor signal transducers (Cheval et al., 2013; Lecourieux et al., 2006). In pepper, transient over-expression of CaM1 activates the production of ROS and nitric oxide, and also induces the expression of defence-related genes (Choi et al., 2009). Furthermore, the gene silencing of *APR134*, a CML in tomato, results in the suppression of HR, while the over-expression of its ortholog CML43 in *Arabidopsis* accelerates the HR response (Chiasson et al., 2005). Recent studies have also highlighted the role of CaM as a positive and negative regulator of SA-mediated defence gene expression, required for both PTI and ETI. Transgenic plants with a mutation in *CBG60g*, a CaM binding transcription factor (TFs), showed defects in *isochorismate synthase 1* (*ICS1*) gene expression, and enhanced susceptibility to *P. syringae* (Wang et al., 2009). In contrast, *enhanced disease susceptibility 1* (EDS1), a key regulator of SA-mediated defence, is negatively-regulated by CaMTA3; plants lacking CaMTA3 show elevated levels of SA and induced expression of EDS1 and SA-mediated defence genes (Du et al., 2009). These data suggest CAM and Ca^{2+} dependent protein kinase may play a critical role in the regulation of SA-mediated defence and induction of HR, two significant components of plant innate immunity.

1.9.2 ROS-mediated defence

Rapid accumulation of ROS after pathogen recognition is one of the earliest events in the plant response and is referred to as the oxidative burst. ROS has been implicated in several tiers of plant resistance, including the induction of defence gene expression, cell wall lignification, phytoalexin accumulation, cellulose deposition and SAR. NADPH oxidase and respiratory burst oxidase homologues (RBOHs) are involved in ROS production. Studies in *Arabidopsis* have identified various mechanisms regulating the RBOHs protein homologues, including protein phosphorylation, mitogen activated protein kinase (MAPK) (Lin et al., 2009), Ca^{2+}, CDPKs (Drerup et al., 2013; Dubiella et al., 2013), and phospholipase C and D (PLC and PLD) (Jakubowicz et al., 2010; Zhang et al., 2009). Phospholipase and its lipid product phosphatidic acid (PA) play an integral role in abscisic acid (ABA) dependent ROS production in guard cells, which results in stomatal closure, a key feature of plant basal defence (Zhang et al., 2009). Recent studies have also shown CDPK-mediated phosphorylation of the

respiratory burst oxidase homologues D (RBOHD) during plant-pathogen interactions. In potato, CDPK4 and CDPK5 are involved in RBOH-dependent ROS production (Kobayashi et al., 2007), suggesting that ROS induction is downstream of Ca^{2+} influx.

In addition to RBOH-dependent ROS, peroxidases (Prx) also play a key role in ROS production (Baxter et al., 2014). In *Arabidopsis*, peroxidases *prx 33* and *prx 34* are major contributors of fungal cell wall and bacterial pathogen-induced ROS production (Baxter et al., 2014; O'Brien et al., 2012). Peroxidase is also involved in the deposition of callose and defence gene expression. When compared to the *rbohD* mutants, plants with mutations in *prx* exhibit stronger down regulation of defence gene expression and reduced callose deposition, suggesting that peroxidase-dependent ROS production may be required for active induction of the defence response (O'Brien et al., 2012; Wrzaczek et al., 2013).

Cellular ROS are tightly regulated by a variety of redox (reduction and oxidation) regulating proteins. Glutaredoxin and thioredoxin are two central players in mediating redox regulation; this is primarily due to their ability to catalyse disulphide bonds (Pieterse et al., 2012; Spoel & Loake, 2011). Studies in *Arabidopsis* have confirmed the role of these redox proteins in SA signalling (Ndamukong et al., 2007; Tada et al., 2008). In SA-induced cells, the conversion of non-expression of PR1 (NPR1) from an oligomer to a monomer is catalysed by thioredoxin (Trx H5), as indicated by a mutation in thioredoxin compromising NPR1-mediated disease resistance (Tada et al., 2008). Whereas glutaredoxin (Grx480) has been identified to interacted with the TGAs (TGACG sequence-specific binding protein, acting downstream of NPR1), overexpression of Grx480 results in the repression of plant defensin 1.2 (*PDF1.2*), characteristic of JA-ET-inducible defence responses against necrotrophic pathogens (Ndamukong et al., 2007; Zander et al., 2014).

1.9.3 Plant hormones-mediated defence

Downstream of ROS, plant hormones play an integral part in activating defence-related signalling networks (Herrera-Vásquez, Salinas, et al., 2015). The induction of these hormones

during microbial invasion is often at the cost of plant development processes. In addition to their activation, the cross-talk between the hormonal pathways and the timing of their interaction play a central role in determining the plant response to the invading pathogen (Robert-Seilaniantz et al., 2011). JA and SA have been recognized as major defence hormones for some time. More recently, however, ethylene (ET), auxin (AU), ABA, gibberellic acid (GA), cytokinins, brassinosteroids and nitric oxide have also been shown to play roles in modulating plant defence responses (Pieterse et al., 2009; Pieterse et al., 2012). Complex cross communication between plant hormones and their roles in plant defence are illustrated in Figure 1.4. In this chapter, only the three hormones (i.e. JA, ET and SA), considered to be important for defence against necrotrophic pathogens, are explained in detail.

1.9.3.1 Jasmonic acid-mediated defence response

JA and its structurally-related metabolites (jasmonate) play a vital role in plant growth and development, mediating growth inhibition, senescence, tendril coiling, flower development, leaf abscission and plant response to abiotic and biotic stress (Wasternack, 2007). JA is derived from the fatty acids linoleic or linolenic acid via the oxylipin biosynthesis pathway. Conversion of linolenic acid to 12-oxo-phytodienoic acid is a multi-step enzymatic process involving lipoxygenase (*lox*), allene oxide synthase (*aos*), allene oxide cyclase (*aoc*) and 12-oxo-phytodienoate reductase 3 (*12-opr3*) (Schaller, 2001). Upon JA synthesis, JA is either metabolized to methyl jasmonate (MeJA), via JA carboxyl methyl transferase (*JMT*) (Staswick & Tiryaki, 2004) or is conjugated to isoleucine in a reaction catalysed by JA-amino acid synthase (*JAR1*). This reaction results in the synthesis of biologically active Jasmonyl Isoleucine (JA-Ile) (Fonseca et al., 2009). To date, JA-Ile is the only known bioactive form of the JA hormone, inducing the interaction between the F-box Coronatine insensitive 1 (COI1) and Jasmonate Zim domain protein (*JAZ*) (Fernández-Calvo et al., 2011; Niu & Figueroa, 2011; Sheard et al., 2010). The JAZ protein in conjunction with COI1 acts as the co-receptor complex of JA-Ile. Binding of JA-Ile results in the ubiquitination and degradation of the JAZ protein. JAZ is a repressor of TFs such as *MYC2* (Niu & Figueroa, 2011). In JA stimulated cells, the interaction between JAZ and *MYC2* is broken, resulting in the activation of the JA signalling pathway and JA-responsive genes (Memelink, 2009).

Though *MYC2* was first identified as a direct target of the JAZ repressor, since then many other JAZ interacting TFs have been identified including ethylene insensitive3 (*EIN3*) and ethylene insensitive-like 1 (EIL1) (Zhu et al., 2011). Indeed, in *Arabidopsis*, two branches of the JA signalling pathway have been identified, the *MYC2* branch and the *ERF* branch. Studies to date have confirmed the mutually antagonistic regulation between *MYC2* and the *ERF* branch of JA signalling. The *MYC2* branch includes a JA-responsive marker gene vegetative storage protein2 (*VSP2*), while the *ERF* branch is characterised by *PDF1.2* (Pieterse et al., 2012). Activation of the *ERF* branch of JA signalling requires coordinate activation of JA and ET signalling and is required for activation of defences against necrotrophs. In contrast, *MYC2* mediated JA signalling is required for defence against insects and herbivores and also plays a role in priming of distal tissues for enhanced pathogen defence (Kazan & Manners, 2012; Lorenzo, Piqueras, Sanchez-Serrano, et al., 2003).

Figure 1.4: Cross-communication of plant hormones during plant-pathogen interactions and the impact of phytotoxin coronatine on host defence response (Pieterse et al., 2009). A solid line represents negative regulation and a dotted line with a purple star represents positive regulation.

1.9.3.2 Ethylene-mediated defence response

Ethylene, being a diffusible phytohormone, plays a critical role in integrating developmental signals such as seed germination, fruit ripening, abscission and senescence and response to biotic and abiotic stress (Yang & Hoffman, 1984). Adverse biotic and abiotic stress induces ET synthesis, which in turn regulates a wide range of genes involved in wound signalling and pathogen defence. ET biosynthetic pathways comprise of three enzymatic steps which are catalysed by S-adenosyl-L-methionine (SAM), ACC synthase (ACS) and ACC oxidase (ACO) (Wang et al., 2002). Upon ET perception, receptors including ETR1, ETS1, ETR2, ETS2 and EIN4 are inactivated resulting in the activation of ethylene insensitive 2 (*EIN2*). This activation

initiates ET signalling. *EIN2* is a central player in the ET signalling cascade. Studies in *Arabidopsis* have identified that the response of the *EIN2* mutant during pathogen attack is generally parallel to the pattern observed in the *jar1-1* and *coi1* mutants and is defective in the ET-mediated defence response (Alonso et al., 1999; Merchante et al., 2013). Furthermore, activation of *EIN2* results in the cleavage and translocation of EIN2 into the nucleus for further stabilisation of EIN3 (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). EIN3 regulates the expression of ET-responsive TFs including *ERF1* and ORF59 (Octadecanoid-Responsive *Arabidopsis* AP2/ERF59), which in turn regulate the expression of defence genes including *PDF1.2* and basic chitinase *(chiB*) (Solano et al., 1998; Tsuda & Somssich, 2015; Zander et al., 2014).

1.9.3.3 Salicylic acid-mediated defence response

SA plays a major role in the plant defence against biotrophic/hemibiotrophic pathogens (Glazebrook, 2005; Pieterse et al., 2012; Robert-Seilaniantz et al., 2011). ICS1 and phenylalanine ammonia lyase (PAL) are the pathways involved in the production of this phenolic compound, and both pathways use chorismate (the end compound of shikimate pathway) to produce SA (Dempsey et al., 2011; Seyfferth & Tsuda, 2014). SA biosynthesis in plants is triggered downstream of both PTI- and ETI-triggered defence responses or on recognition of PAMPs or effectors (Tsuda et al., 2008; Tsuda & Katagiri, 2010; Tsuda et al., 2013). During PTI and ETI (initiated by TIR-NBS-LRR type *R* proteins) mediated SA signalling, EDS and Phytoalexin Deficient 4 (PAD4) play a major role in the onset of SA-related hormone response(Wiermer et al., 2005). However, when ETI is initiated by CC-NBS-LRR type *R* protein, Non Race-Specific Disease Resistance 1 (NDR1), triggers the onset of SA biosynthesis (Bernoux et al., 2011).

The downstream activation of the SA-mediated resistance response is controlled by NPR1 (Cao et al., 1997). In SA-induced cells, NPR1 is translocated to the nucleus, where NPR1 monomers interact with the TGAs, that bind to the SA-responsive genes including PR1 resulting in their induction (Fan & Dong, 2002). Following their induction, NPR1 is ubiquitinated and degraded by the proteasome (Spoel et al., 2009; Yan & Dong, 2014). The

PR-1 gene is a well characterised marker for SA-responsive gene expression that is involved in pathogen related defence (Pieterse et al., 2012; van Loon et al., 2006). *PR* gene activation is not always SA-dependent, however, and the *PR* genes activated upon pathogen attack are not always similar to the *PR* genes activated by exogenous application of SA or its functional analogue (Pieterse & van Loon, 1999).

1.9.4 Transcriptional factors involved in plant defence

In addition to defence gene regulation through hormone-mediated signalling, transcriptional reprogramming is a major feature of plant immunity and is governed by various TFs and co-regulatory proteins associated with the transcriptional complex. Several TF families have been associated with defence signalling and the transcriptional reprogramming. However, in this thesis, only TFs known to be involved in plant-pathogen interactions will be discussed in detail.

The transcription of the *WRKY* gene is strongly induced in response to many environment clues including pathogen infection. In *Arabidopsis,* eight *WRKY* genes (*WRKY18*, *WRKY38*, *WRKY53*, *WRKY54*, *WRKY58*, *WRKY59*, *WRKY66* and *WRKY70*) were identified as key regulators of SA-mediated defence responses (Ishihama & Yoshioka, 2012). *WRKY18* and *WRKY40*, closely related to *WRKY1/2*, negatively regulate the expression of EDS1 (SA pathway) and positively regulate JA-mediated defence signalling (Pandey et al., 2010). Furthermore, *WRKY18* and 40 exert negative control on camalexin production. These responses are important in response to biotrophic pathogens. In contrast, *WRKY33* plays a significant role in the plant defence against necrotrophic pathogens by inducing camalexin biosynthesis (Mao, Meng, et al., 2011; Petersen et al., 2010). In *Arabidopsis*, *WRKY33*, the closest to tobacco *WRKY8*, was identified as the substrate for MAPKs; MPK3 and MPK6, and is involved in defence against *B. cinerea*, necrotrophic pathogen (Lai & Mengiste, 2013; Mao, Meng, et al., 2011).

In addition to *WRKY* TFs several *MYB* TFs have been implicated in hormone signalling, primary and secondary metabolite biosynthesis and regulation of plant responses to biotic and abiotic stress (Ambawat et al., 2013; Dubos et al., 2010; Liu et al., 2015). The R2R3-MYB transcription factor plays a significant role in the resistance against necrotrophic pathogens (Vailleau et al., 2002). In cotton, *MYB108* TF participates in the defence against *Verticillium dahlia* by interacting with CML11 (Calmodulin-like protein 11) (Cheng et al., 2016). *MYB12* is also involved in flavonoid biosynthesis and is induced by UV-B. Recent studies have shown flg22-mediated suppression of *MYB12* and activation of *MYB4*. However, the signalling cascade related to flg22-meditated down regulation of *MYB12* remains unknown (Schenke & Cai, 2014). *MYB* transcription factor also play a vital role in the secondary metabolite synthesis including phenylpropanoids, flavonoids, isoflavonoids, and phenolic acids biosynthesis. In *Arabidopsis*, *MYB11*, and *MYB12* are involved in the induction of the genes related to the flavonoid biosynthesis including chalcone synthase, chalcone isomerase, flavanone 3 hydroxylase and flavonol synthase (Luo et al., 2008; Misra et al., 2010; Pandey et al., 2012; Pandey et al., 2014). *MYB* TFs have also been associated with lignin biosynthesis (Liu et al., 2015). In *Arabidopsis* SND1, secondary cell wall associated protein, interact with *MYB83* and *MYB46*, and subsequently induces the expression of *MYB58*, *MYB63* and *MYB85* involved in the lignin biosynthesis (Liu et al., 2015; Zhong et al., 2008; Zhou et al., 2009).

1.9.5 Secondary Metabolism

Plants produce a diverse range of natural compounds known as secondary metabolites. Unlike primary metabolites, these compounds are not essential for the normal molecular processes in the cell but are required under certain environmental stress conditions. Plants from related families produce related secondary metabolites, for example, *Solanaceous* plants produce sesquiterpene while *Leguminosas* produce isoflavonoids (Dixon, 2001). The diversity in the production of secondary metabolites in plants is in part due to the need for an improved defence against microbial attack or insect infestation (Dixon, 2001). The production of these diverse secondary metabolites is coordinated through a complex signal

transduction pathway and is linked to pathogen recognition by the host receptors (Jones & Dangl, 2006).

Studies in cotton have shown an increase in the cellular concentration of sesquiterpenoid in leaves during infection with the bacterial pathogen *Xanthomonas campestris* pv *malvacearum* (Essenberg et al., 1992; Pierce et al., 1996). The concentration of these phytoalexins in the cells surrounding the infection site was consistently higher than the concentration required to effectively inhibit growth of the pathogen *in vitro* (Davis et al., 1996; Pierce et al., 1996). In *Arabidopsis*, flowers of transgenic plants lacking (E)-β-caryophyllene synthase, a sesquiterpene synthase, were more susceptible to *P. syringae* bacterial growth. In contrast, ectopic expression of (E)-β-caryophyllene synthase results in enhanced resistance to the same pathogen, suggesting that this sesquiterpene released by the flowers may act as a bacterial growth inhibitor (Huang et al., 2012). However, the exact mechanism employed by sesquiterpene to exert its antimicrobial property remains unknown.

1.10 Understanding plant-pathogen interactions and its impact on improved disease management in plants

Conventional plant breeding has been used to enhance the traits of crops since man started to cultivate plant species. One of the traits often targeted has been disease resistance, due to the significant impacts pests and diseases have on yield and quality. Indeed, even at its simplest, the recognition that some variants of a plant species have a greater ability to combat a specific pathogen than others has enabled the breeding of disease resistance. Knowledge of plant-pathogen molecular interactions and the mechanisms used by plants to combat disease have provided an even greater understanding of how pests and plants coexist. For example, we now understand the interactions of 'avirulence' effectors and R proteins, which has enabled more sophisticated methods of breeding to be introduced by considering the gene for gene hypothesis. Whole genome sequencing of both host and pathogen have also showed the complexity of the interactions between effectors and R proteins and

demonstrated the importance of developing more durable resistance by introducing multiple resistance genes.

Blackleg and tuber soft rot continue to cause economic losses to potato production as they are difficult to manage through conventional farm practices. Thus, it is likely that our greatest opportunities to combat these diseases will come through the development of resistant cultivars. To date, progress towards identifying resistance or tolerance in potato to SRE has been limited. For instance, Kubheka et.al., (2013) showed that some cultivars used in South Africa have greater tolerance to blackleg caused by *Pbr*. In addition to quantitative resistance breeding, genetically modified (GM) plants have shown promise, with several studies confirming the resistance of GM potato plants to *Pectobacterium* spp. For example, transgenic potato plants expressing acyl homoserine lactones (AHLs) from *Bacillus* showed high levels of resistance to *Pcc*, in which either the symptoms were blocked or were significantly reduced (Dong et al., 2001). Likewise, ectopic expression of 5-O-glucosyltransferase in transgenic potato plants resulted in at least a two-fold reduction of disease symptoms in transgenic tubers when compared non transformed plants (Lorenc-Kukuła et al., 2005). In New Zealand, Mohan et.al.,(2014) showed that induced expression of GSL2 led to greater resistance to soft rot and blackleg caused by *Pba*.

Despite progress in developing GM plants with resistance to SRE, there is presently no appetite to use GM potato crops to manage these (or other) diseases in New Zealand. As an alternative to transgenic plants, however, a cisgenic approach has been suggested, where the recipient cultivar is modified with resistance genes from the same sexually compatible species. To date though, no resistance genes have been identified to control soft rot and blackleg disease so it is likely that a complex expression of genes would be involved in resistance. Hence, further studies on *Pectobacterium*-potato interactions at molecular level could provide novel insights into the plant induced defence response and thereby provide candidate genes for further resistance breeding.

1.11 Key objectives

Little information is currently available on the genetic responses of potato to infection with SRE. Yet, this information is imperative in order to gain a greater understanding of the interactions between these pathogens and their host. The availability of the whole genome sequence (*S. tuberosum* group Phureja DM1-3) (Potato Genome Sequencing Consortium, 2011) enables us to now use sophisticated molecular techniques such as genotyping-by sequencing (GBS) or transcriptomics to map resistance traits or to understand the global responses in the host that might confer resistance to SRE.

In this thesis, RNA-seq was used to examine the transcriptional response of potato to two SRE taxa during a susceptible interaction, in order to understand the early innate immune responses in this plant, and to set up the pipelines for future analysis of resistant lines identified in the Plant & Food Research breeding programme (data unpublished). The opportunity was also taken to investigate how CFA, an important virulence factor in these SRE, affects their interactions with the host. The role of CFA and COR has been studied extensively in *P. syringae* but given that *Pectobacterium* spp. are necrotrophic or hemibiotrophic pathogens and possess only the CFA cluster, questions remain as to (i) whether CFA in these *Pectobacterium* spp. effects similar host defence pathways, and (ii) if the host defence responses in tubers are similar or different to those in above ground organs? With an aim to answer these questions, the key defence responses employed by potato tubers during a susceptible interaction with SRE were examined and compared to those that occurred in the presence of exogenous CFA or when CFA was no longer synthesized by the pathogen.

Chapter 3 is divided into two sections: (i) the identification of the most appropriate time points for transcriptome analysis (ii) followed by the identification of the most robust and reliable software package available for differential gene expression analysis.

Chapter 4 examines the key host defence pathways or gene groups differentially expressed during the potato-*Pectobacterium* interaction, following the hypothesis that both pattern and

effector-triggered defence pathways relating to necrotrophic/hemibiotrophic pathogens should be differentially expressed during soft rot infection.

Chapter 5 aimed to evaluate the impact of CFA on tubers during the potato-*Pectobacterium* interaction. This section of the thesis was guided by two questions: (i) Like COR/CFA in *P. syringae,* does the application of exogenous CFA impact key plant defence pathways in potato tubers? (ii) Does the loss of HAI2, encoding the CFA gene cluster, impact the plant defence response during a *Pectobacterium*-potato interaction?

Chapter 6 examines the expression and the production of CFA and CFA conjugates in potato during *Pectobacterium* infection, following the hypothesis that, as in *P. syringae* and *S. scabies*, conjugation of CFA to an amino acid is essential for the bioactivity of CFA in potato tubers. This should characterise the CFA conjugate produced by *Pectobacterium* during infections in potato plants.

Chapter 2
Materials and Methods

2.1 General methods

2.1.1 Bacterial culture conditions

Pectobacterium strains (Table 2.1) were routinely cultured in Luria Bertani (LB) broth (Miller, 1972) at 200 rpm in a shaking incubator at 28°C for 24 h, unless otherwise stated. For long term storage, 1 volume of an overnight culture was mixed with an equal volume of 20% glycerol and stored at -80°C. To isolate individual colonies, bacterial cultures were revived from the frozen stock by streaking onto LB plates supplemented with 1.6% (w/v) agar. Bacterial strains were obtained from the International Collection of Microorganisms from Plants (ICMP), Landcare Research, New Zealand and Scottish Crop Research Institute (SCRI, now James Hutton Institute), Dundee, UK.

To identify the optimal conditions for CFA production, *Pectobacterium* strains were also grown at 28°C or 18°C in either Minimal media (MM) or Minimal media with M9 salts (MM+M9) (Table 2.2) supplemented with either 10 mM sucrose, fructose or glucose. When necessary, these media were supplemented with 5% v/v tuber extract. Tuber extracts were prepared by grinding 200 g potato tubers 'Ilam Hardy' with 1 l water, in a blender. The tuber debris was removed by centrifugation at 16000 *g* for 10 min. The supernatant was filter sterilised (0.2 µm) (Merck, Germany) (Mattinen et al., 2007).

Table 2.1: Bacterial strains used in this study.

Strains	**Description**	**Source**
Pba SCRI1043	Wild-type strain that causes soft rot of potato tubers and blackleg disease of potato stems	(Bell et al., 2004)
Pba SCRI1043ΔHAI2	*Pba* SCRI1043 strain with deletion of HAI2, engineered through CRISPR-Cas targeting	(Richter et al., 2014; Vercoe et al., 2013)
Pbr ICMP19477	New Zealand wild-type strain that causes soft rot of tubers and blackleg disease of plants.	(Panda et al., 2012)

Table 2.2: Media composition

Media	**1 l**
Minimal Medium	
50 x Phosphate buffer (pH 6)*	20 ml
1 M Ammonium sulphate ($(NH_4)_2SO_4$)	13.3 ml
1 M Magnesium sulphate ($MgSO_4$)	10 ml
50 mM sugar source	200 ml
Water	756.70 ml
Minimal Medium (M9 salts)	
5 x M9 salts (Sigma-Aldrich)*	200 ml
1 M Magnesium sulphate ($MgSO_4$)	2 ml
1 M Calcium chloride ($CaCl_2$)	0.1 ml
50 mM sugar source	200 ml
Water	597.9 ml
Huynh's Minimal Medium (HMM) (Huynh et al., 1989)	
1 M Phosphate buffer (pH-6)	50 ml
1 M Ammonium sulphate ($(NH_4)_2SO_4$)	1 ml
1 M Magnesium chloride ($MgCl_2$)	17 ml
5 M Sodium chloride (NaCl)	0.34 ml
50 mM Sugar source	200 ml
Tuber extract (section 2.1.1)	50 ml
Water	681.66 ml

***5 x M9 salt solution**

To prepare 5 x M9 salt solution 56.4 g of M9 salts (Sigma Aldrich) was dissolved in sterile distilled water (SDW) and made up to 1 l; sterilized by autoclaving.

***50 x Phosphate buffer**

To prepare 50 x phosphate buffer, 2 M of Dipotassium hydrogen phosphate (K_2HPO_4) and 0.5 M of Potassium dihydrogen phosphate (KH_2PO_4) were dissolved in SDW and made up to 1 l; sterilized by autoclaving.

2.1.2 Preparation of plants and bacterial inocula for pathogenicity assays

2.1.2.1 Soft rot assay

Bacterial cultures were grown overnight in LB broth at 200 rpm in a shaking incubator at 28°C. Cells were harvested by centrifugation at 200 *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in 1 ml of sterile 10 mM $MgCl_2$. Inoculum concentration was adjusted to 10^6 colony forming units (CFUs), by measuring the optical density at 600 nm using a spectrometer and by adjusting the volume to bring it toOD_{600}= 0.8.

Prior to inoculation, potato tubers were washed in tap water, air-dried and weighed. Using a cork borer, uniform bores (approximately 3 mm diameter by 10 mm deep) were then made in the tubers (1 bore per tuber) 10 µl (10^4 cells per inoculation site) of the overnight inoculum was inoculated into the hole using a pipette. The tuber plug was replaced and sealed using petroleum jelly. Tubers were placed in a plastic container layered with paper towels soaked in sterile water (to maintain humidity) and incubated in a dark room at 22°C for 1 week. As a negative mock-inoculated control, tubers were inoculated with 10 mM $MgCl_2$. After incubation, the rotten tissue was removed by washing, and the tubers were re-weighed after blotting dry with a paper towel.

2.1.2.2 Blackleg assay

Potato tubers were allowed to sprout at room temperature for approximately 2 weeks. The sprouted tubers were potted in sterile potting mix and the plants were grown for approximately three weeks in a controlled growth chamber, with a 16 h photoperiod at 22°C and 80% humidity. When they reached 20-25 cm in height, stems were inoculated with 10 µl of an overnight culture (equivalent to 10^4 cells per inoculation site) at the lower junction of the stem and the second leaf from the top of the plant. Bacterial inocula were prepared as described in section 2.1.2.1. The inoculation sites were wrapped with film to seal the wound. As a mock-inoculated control, plants were injected with 10 mM $MgCl_2$.

2.2 Transcription studies using RNA sequencing

2.2.1 Response of potato tubers to *Pectobacterium* and CFA mutants

Potato tubers ('Summer Delight') were prepared as described in section 2.1.2.1. Ten tubers were inoculated with 10 µl of overnight culture (containing approximately 10^4 cells per inoculation site) of either *Pbr* ICMP19477, *Pba* SCRI1043, *Pba* SCRI1043ΔHAI2 or 10 mM $MgCl_2$ as described previously in section 2.1.2.1. Susceptibility to soft rotting was assessed by calculating the weight of tissue lost after removing the rotten tissue (Wright et al., 1991). The percentage of weight loss (i.e. 100*(initial weight – final weight)/initial weight) was analysed using a binomial generalized linear model approach (McCullagh & Nelder, 1989), with a logit link. Differences between strains and other contrasts were assessed with F-tests within the analysis of variance conducted as part of the analysis. The analyses were carried out with GenStat (GenStat Committee, 2011).

2.2.2 Dose response curve to exogenous CFA

To identify the dose response curve of potato tubers to exogenous CFA, tubers of 'Summer Delight' were treated with 1 µl to 20 µl droplets of exogenous CFA (Robin Mitchell, Plant & Food Research, New Zealand) (20 nmol to 400 nM concentration per inoculation site, suspended in SDW) using the same procedure as mentioned in section 2.2.1. The tuber plug was replaced and sealed using petroleum jelly and the tubers were placed in a plastic container and incubated at 22°C. Five replicates were used for each treatment. Tubers were evaluated for symptoms 5 days post inoculation (dpi). As positive controls, tubers were inoculated with 10 µl (10^4 cells) of an overnight culture of *Pba* SCRI1043. A mock-inoculated control was generated by inoculating tubers with 10 mM $MgCl_2$.

2.2.3 Measurement of bacterial growth in potato tubers

Potato tubers ('Summer Delight') were prepared as described in section 2.1.2.1. Ten µl of an overnight culture (containing approximately 10^4cells per inoculation site) of *Pbr* ICMP19477, *Pba* SCRI1043 or *Pba* SCRI1043ΔHAI2 were inoculated into each tuber as described previously in section 2.2.1. Tubers were incubated as described previously in section 2.1.2.1. The bacterial population in each tuber was measured at 0 and 12 hpi, and 1, 3, 5 and 7 dpi. Each time point was represented by 3 tuber replicates. The tubers were sampled around the inoculation site, and were homogenised using a sterile micropestle. A 100 µl aliquot of the lysate was resuspended with 900 µl of sterile water, and a serial dilution of each replicate was prepared and plated on LB agar plates (1 plate per dilution). Tubers inoculated with 10 mM $MgCl_2$ were used as negative controls. The CFUs in each plate were counted and the data for different treatments were analysed statistically.

The data for all counted plates were included in the statistical analysis, with data for all strains compared in a single analysis. The standard approach for counts is a Poisson generalised linear model (GLM, McCullagh & Nelder, 1989). However, for this experiment, there were multiple plates per tuber, with separate plates for different serial dilutions from any given tuber. Therefore, the data were analysed using an extension of the Poisson GLM that allowed adjustments for individual tubers to be included (as a random effect) and also an adjustment for the dilution factor for each plate. The adjustment included the dilution factor as a parameter-less offset (McCullagh & Nelder, 1989); that is, the count per plate was the underlying count for the tuber, divided by the dilution factor. The counts were analysed with a Poisson-gamma hierarchical GLM (Lee et al., 2006) with logarithmic links, and an offset of log(1/dilution). Times were included as a fixed effect and tubers as a random effect. In addition, the random variation was estimated separately for tubers inoculated with different strains. The analysis was carried out with GenStat (GenStat Committee, 2011).

2.2.4 RNA sequencing experimental design

To study the transcriptional response in potato tubers to bacterial infection and to exogenous CFA, a replicated experiment was designed as illustrated in Table 2.3. Bacterial inocula for infection of potato tubers were then prepared as described in section 2.1.2.1. Potato tubers of 'Summer Delight' were inoculated with 10 µl of overnight cultures (containing approximately 10^4 cells per inoculation site) of either *Pba* SCRI1043, *Pba* SCRI1043ΔHAI2 or *Pbr* ICMP19477 as described previously in section 2.1.2.1. For CFA treatment, potato tubers were inoculated with 10 µl of exogenous CFA (20 nmol) suspended in SDW (Robin Mitchell, Plant & Food Research, New Zealand). The dose of CFA used in the experiment was determined by an initial dose response curve in tubers (as described in section 2.2.2). Potato tubers were also inoculated with 10 mM $MgCl_2$ as mock-inoculation controls while non-inoculated tubers were set up as negative controls.

2.2.4.1 Potato tissue sampling

Tissues from the tubers were sampled around the site of inoculation at 6, 12 and 24 hpi. These sampling times were determined to represent the initial stages of infection (lag, exponential and stationary phase) by analysis of bacterial growth in tubers. Tissue from each tuber was sampled by creating 8-10 bores around the site of inoculation (Figure 2.1). Tissues from three tubers were pooled together to represent 1 biological replicate. Each treatment and the mock-inoculated control were represented by three biological replicates for each time point. The non-inoculated control was represented by three biological replicates. The samples were immediately flash frozen using liquid nitrogen and stored at -80°C before freeze drying.

For freeze drying the tuber material was placed into perforated bags and frozen at -22°C overnight. Bags were loaded into aluminium trays (500mm L X 200mm W X 50mm D) and placed into the vacuum chamber. The condenser operating temperature was -30°C. The chamber was evacuated using an Edwards RV 12 Hi Vac pump and run for 96 hours by which

time the vacuum pressure had dropped to around 0.4mBar (0.3 Torr). The vacuum was released and the freeze dried plant material stored at -80°C until further use.

Table 2.3: A list of treatments and biological replicates for RNA sequencing.

Treatment/time point	**0 hpi**	**6 hpi**	**12 hpi**	**24 hpi**
Pba SCRI1043	-	3	3	3
Pba SCRI1043ΔHAI2	-	3	3	3
Pbr ICMP19477	-	3	3	3
CFA (200nM)	-	3	3	3
Mock-inoculation (10 mM $MgCl_2$)	-	3	3	3
Non-inoculated	3	-	-	-

Note:-The numbers in the table represent the number of biological replicates for each treatment at individual sampling times. Each biological replicate represents a pooled sample of three tubers.

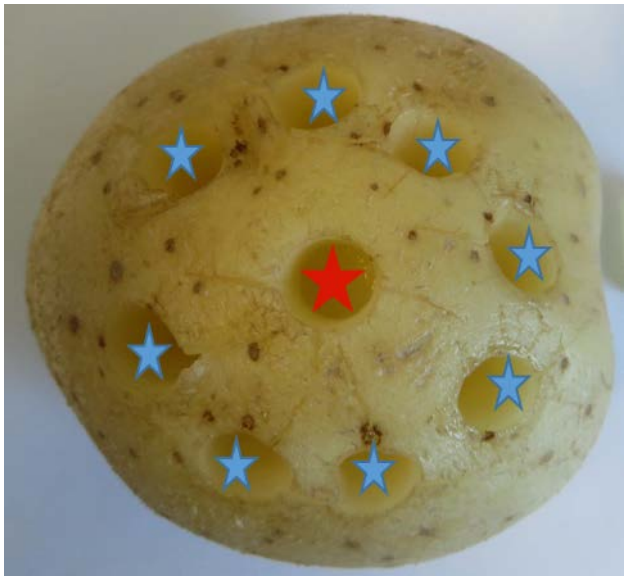


Figure 2.1: Location of tissue sampling sites for total RNA extraction.
Bacterial inocula or exogenous CFA were inoculated into potato tubers (Red star). Tissues were taken from 8-10 bores (Blue star) distal to the site of inoculation.

2.2.5 Isolation of total RNA from potato tubers

Total RNA from freeze dried potato tubers was isolated using a Lithium chloride (LiCl) precipitation method (Barlow et al., 1963). For each sample, 7 ml of extraction buffer (Table 2.4) was added to a 15 ml falcon tube, followed by addition of 7 ml of phenol (pH 4.6-5.6). The resulting extraction buffer was incubated in a 80°C water bath for 15-30 min. Approximately 100 mg of freeze dried tuber tissue was ground in liquid nitrogen using a mortar and pestle prior to the addition of 14 ml of hot extraction buffer to the sample. The sample mixture was transferred into a 15 ml falcon tube and mixed using a vortex for 2-5 s. 10 ml of SDW was added to the sample, and again the content was vortexed for 1-2 min followed by addition of 15 ml of chloroform: isoamyl alcohol (24:1). After repeated vortexing for 1-2 min, the samples were centrifuged at 4°C for 20 min at 20817 *xg*. The upper aqueous layer was transferred into a 50 ml falcon tube containing equal volumes (16 ml) of 4 M LiCl previously incubated at -20°C. The contents were mixed thoroughly by shaking, and the tube was incubated at -80°C overnight. After incubation, the tube was thawed on ice and was centrifuged at 4°C at 20817 *xg* for 40 min. The supernatant was discarded, and the pellet was resuspended in 5 ml SDW, 0.1 volume of 3 M sodium acetate (NaOAc) (pH 5.2) and 3 volumes of absolute ethanol. The sample was then incubated at -80°C for at least 1 h. After incubation, the sample was centrifuged at 4°C at 20817 *xg* for 40 min. Ethanol was discarded, and the pellet was air dried. The total RNA was resuspended in 500 µl of RNase-free water. The RNA was then purified, and genomic DNA contamination was removed using the SV total RNA isolation kit (Promega) according to the manufacturer's instructions.

Table 2.4: Extraction buffer used for total RNA extraction from potato tubers

Reagent	**100 ml**
1 M Tris-HCl (pH 8.0)	10 ml
4 M LiCl	2.4 ml
0.5 M EDTA	2 ml
SDS 20 % (w/v)	5 ml
Water (SDW)	80.6 ml

2.2.5.1 DNase treatment

In addition to the on-column DNase treatment described above, RNA samples were subjected to DNase digestion using TURBO DNase (TURBO DNA free kit, Ambion). For 10 µg of RNA, 1 µl (2U) of DNase was added to a 50 µl reaction. The reaction mixture was incubated at 37°C for 20 min. After incubation, an additional 1 µl of TURBO DNase was added to the reaction and incubated for a further 20 min at 37°C; enabling the efficient degradation of any DNA contamination. DNase was inactivated by adding 0.1 volume of DNase inactivation reagent. RNA was then purified by following the manufacturer's instructions.

2.2.5.2 RNA quality control

The integrity of the RNA extracted from potato tubers was determined using a Bioanalyzer 2100 (Agilent Technologies). Reagents for the RNA bioanalyzer were prepared based on the manufacturer's protocol. Samples with good quality RNA have a RNA Integrity Number (RIN) ≥ 7, whereas a RIN < 7 indicates significant RNA degradation.

2.2.6 RNA sequencing

Total RNA (~ 1 µg) from each biological replicate was sequenced using the Illumina HiSeq platform. Sequence libraries were prepared from each sample of RNA (a total of 48) according to Illumina instructions by New Zealand Genomics Lab (NZGL). All 48 libraries were normalised, pooled and sequenced across five lanes of illumina HiSeq 2000 yielding up to 225 Million 100 bp paired-end reads per lane. The RNA sequence data was analysed using the software tools listed in Table 2.5.

The general workflow for the RNA-seq analysis is illustrated in Figure 2.2. The raw Fastq output files obtained from the Illumina HiSeq were quality checked and trimmed. The main aim of the analysis was to identify the potato genes that are significantly differentially

expressed (DE) in response to *Pectobacterium* and CFA. This was achieved by mapping the filtered reads from each library separately to the potato reference genome (The Potato Genome Sequencing Consortium, 2011) using the splice junction mapper TopHat2 (Kim et al., 2013) with Bowtie2 (Langmead & Salzberg, 2012). The total reads were also mapped to the reference genome of *Pba* SCRI1043 (Bell et al., 2004) and *Pbr* ICMP19477 (Panda et al., 2015), to confirm the presence of bacterial RNA. HTSeq-count was used to count raw data of the mapped reads to the annotated gene regions. Read counts were further analysed using DESeq2 (Love et al., 2013) implemented in R for differential expression analysis. Genes with fold changes greater than ±2 log2 fold and *p* adjusted values (p_{adj}) less than 0.05 were used for further funtional analysis. For comparative analysis, Cufflinks and Cuffdiff were used for initial DE analysis. For this purpose, the aligned reads were used as input in cufflinks. The resulting differentially expressed genes (DEGs) were filtered using the same log2 fold and p_{adj} (q value) cut-off values as used for DESeq2 analysis. All commandline workflows used for RNA-seq analysis are described in section 2.2.8.

Table 2.5: A list of bioinformatics tools used for analysis of RNA sequencing data.

Tool	URL	Reference
Quality control		
Fastqc (v0.11.2)	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/	(Andrew, 2010)
FastqMcf (v1.04.803)	http://code.google.com/p/ea-utils/wiki/FastqMcf	N/A
Hard TRIM fq2trimmed_extended.pl	in-house (PFR) developed perl script	N/A
Read alignment		
Bowtie2 (v2.2.5)	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml	(Langmead & Salzberg, 2012)
TopHat (v2.0.13)	http://tophat.cbcb.umd.edu/	(Kim et al., 2013)
Count data		
HTSeq-count (v0.6.1p1)	http://www-huber.embl.de/HTSeq/doc/overview.html	(Anders et al., 2014)
Differential expression analysis		
DESeq2 (v 1.6.3)	http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html	(Love et al., 2013)
R studio (v3.1.3)	https://www.rstudio.com/	(RStudio Team, 2015)
R	https://www.r-project.org/	(R Development Core Team, 2011)
Cufflinks & Cuffdiff (v2.2.1)	http://cole-trapnell-lab.github.io/cufflinks/manual/	(Trapnell et al., 2013)
Functional analysis		
Blast2GO (3.3.5)	https://www.blast2go.com/	(Conesa & Gotz, 2008; Conesa et al., 2005)
MapMan (3.3.0RC1)	http://mapman.gabipd.org/web/guest/mapman	(Thimm et al., 2004)

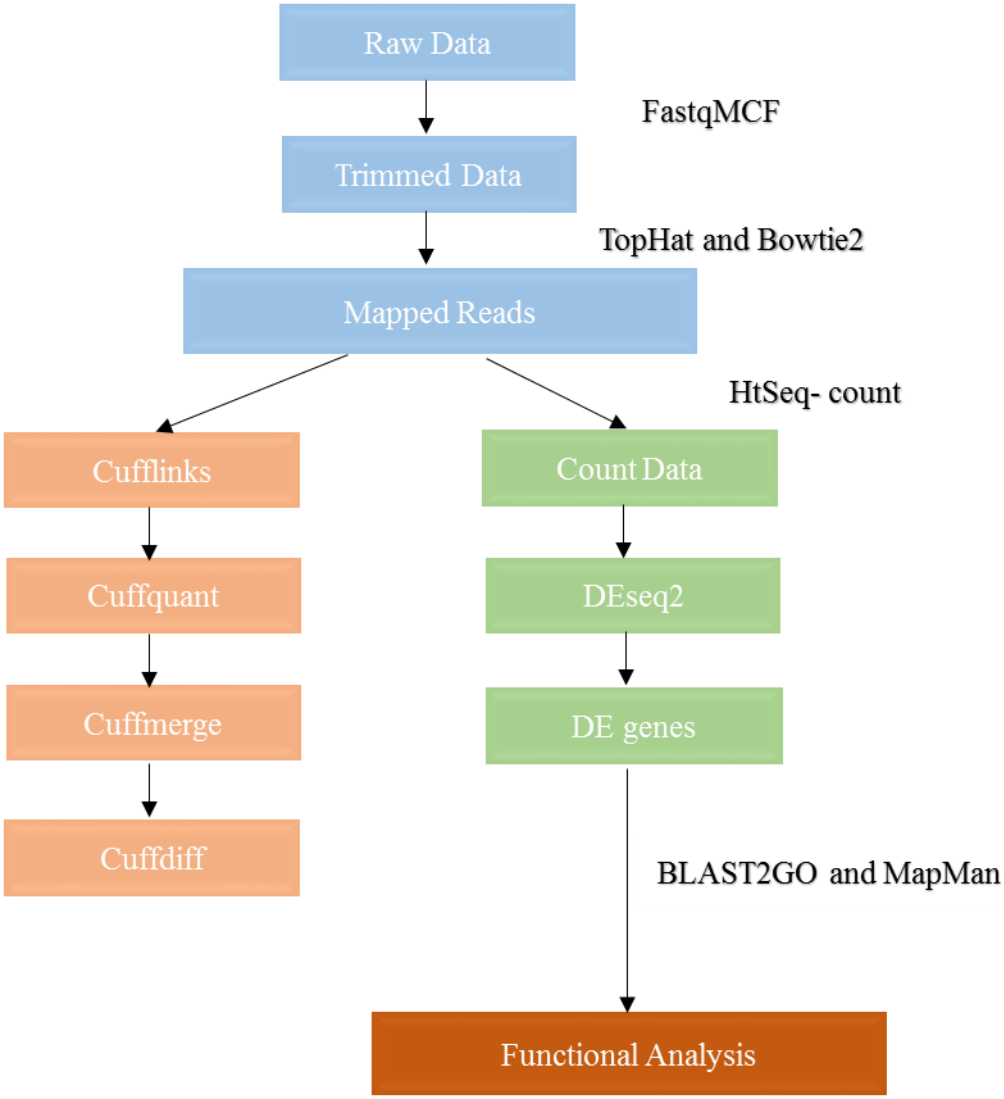


Figure 2.2: Schematic outline of the workflow used in transcriptome analysis.

2.2.7 UNIX command lines for RNA sequencing analysis

2.2.7.1 RNA sequencing quality control and trimming

2.2.7.1.1 FastQC

Raw sequences obtained from NZGL were subjected to initial quality control analysis using FastQC (Andrew, 2010). FastQC provides an overview and graphical summary of the different aspects of the data including per base sequence quality, per sequence quality score, GC content, sequence length, adapter sequence presence and kmer. FastQC analysis was run using default settings.

2.2.7.1.2 FastqMCF

After the initial quality control check, the adapter sequence, reads with poor quality and the Ns from the ends were removed using FastqMCF (https://expressionanalysis.github.io/ea-utils/). During the following trimming steps the RNA-seq pairing was maintained. For effective removal of the adapter sequence the following options were changed from the default setting: -l minimum required length or the remaining read to be retained, -q quality threshold causing basal removal, -t percentage of occurrence threshold before adapter clipping, -C number of reads to use for subsampling. The following script was used for FastqMCF:

```
>fastq-mcf -o clean.sample_R1.fastq -o clean.sample_R2.fastq -l 50 -q 15 -t 0.1
        + -C 1000000 TruSeqAdapters.fasta sample_R1.fastq sample_R2.fastq
```

2.2.7.1.3 Hard TRIM

In addition to FastqMCF, an additional hard TRIM was also performed. For this purpose, 15 bases from the 5' and 10 bases from the 3' end were removed. The following script was used for hard TRIM:

```
>fq2trimmed_extended.pl -c=trim, removeN -5=15 -3=10
        +-f1=clean.sample_R1_001.fastq -f2=clean.sample_R2_001.fastq
```

2.2.7.2 Alignment to the reference potato genome and read counts

2.2.7.2.1 TopHat alignment

The first step in RNA-seq analysis involves mapping filtered reads to the reference genome; this provides information regarding the location of the transcript. TopHat2 (Kim et al., 2013) and Bowtie2 (Langmead & Salzberg, 2012) were used to align the reads to the potato reference genome PGSC v4.03 (Sharma et al., 2013). The following options were specified for effective alignment.

--b2-sensitive:- bowtie 2 option for alignment (default is end-to-end mode)

--no-convert-bam:- Do not convert to bam format

-G:- Supply TopHat with gene model annotation

-p:- number of threads to align (default is 1)

The fastq reads from each lane were merged by samples and aligned to the reference genome using TopHat.

```
>tophat --b2-sensitive --no-convert-bam -o ./sample.tophat
        + -G PGSC_DM_V403_fixed_representative_genes.gff -p 6
        +PGSC_DM_v4.03_pseudomolecules_ALL.fasta sample_R1.fastq
        sample_R2.fastq
```

2.2.7.2.2 Count data using HTSeq-count

To obtain count data for downstream gene level differential expression analysis,the HTSeq-count tool (Anders et al., 2014) was used. HTSeq-count uses the GFF (annotation files) and SAM (alignment files) files to count the number of aligned reads that overlap the exon of each gene. The following options were specified for HTSeq-count.

-s :- a read is considered overlapping with a feature regardless of whether it is mapped to the same or the opposite strand as the feature
-t :- feature type (3rd column in GFF file) to be used
-i :- GFF attribute to be used as feature

The following script was used to obtain the count data for each replicate of each treatment and each time point.

Sort alignment files

```
sort -s -k 1,1 accepted_hits.sam > ./sample.sorted.sam
```

HTSeq-count

```
>python -m HTSeq.scripts.count -s no -t gene -i ID -o sample.out.sam sample.sorted.sam
        +PGSC_DM_V403_fixed_representative_genes.gff >sample_htseq.count
```

2.2.7.3 Differential expression analysis

The primary aim of this thesis was to identify the DEGs in response to one or more treatments over the period of disease progression. The major difficulty with RNA-seq data analysis is the degree of variation between biological samples, and biases introduced during sample preparation, library preparation and sequencing. The differential expression analysis of RNA-seq data consists of three crucial steps: normalisation of counts, parameter estimation of the statistical model and the test for differential gene expression. In this study two well-studied differential expression analysis tools DESeq2 and Cuffdiff were used for normalisation and differential expression testing.

2.2.7.3.1 DESeq2

The DESeq2 package (Love et al., 2013) provides tools to test for differential expression using the negative binomial GLM by estimating dispersion and logarithmic fold changes between the treatments. DESeq2 takes raw read count data as the input and introduces possible sources of bias into the negative binomial distribution model to perform integrated normalization as well as differential expression analysis (Conesa et al., 2016; Love et al., 2014). It also provides methods for differential expression analysis by using shrinkage estimation for dispersion and logarithmic changes. In addition, DESeq2 detects gene outliers within the replicates using Cook's distance. In DESeq2, detection of genes with inconsistent read counts between biological replicates causes the entire gene to be flagged and removed from further downstream analysis (Love et al., 2013). The package uses, pairwise comparisons to estimate the differential expression. In this study, each treatment was compared with the non-inoculated control. The R code used for pairwise comparisons is described below. As an example, the pairwise comparison between *Pba* SCRI1043 (Pba) and the non-inoculated control (NI) is illustrated. HTSeq-count was used as the input for the DESeq2 analysis. The working directory was set using the following script.

```
directory <- "H:/Ph.d/RNA_PhD/Deseq2/Pbavscontrol"
setwd("H:/Ph.d/RNA_PhD/Deseq2/Pbavscontrol")
```

Input data

```
samplefiles <- grep(".count",list.files(directory),value=TRUE) sampleFiles
samplename <- c("1NI_0", "1Pba_06", "1Pba_12", "1Pba_24", "2NI_0", "2Pba_06",
"2Pba_12", "2Pba_24", "3NI_0", "3Pba_06", "3Pba_12", "3Pba_24")
sampletime <- c("0", "06", "12", "24", "0", "06", "12", "24", "0", "06", "12" ,"24")
samplecondition <- c("NI", "Pba", "Pba", "Pba", "NI", "Pba", "Pba", "Pba", "NI", "Pba",
"Pba", "Pba")
sampleTable <- data.frame(sampleName=samplename, fileName=sampleFiles,
condition=sampleCondition, time=sampletime)
ddsHTSeq <-
DESeqDataSetFromHTSeqCount(sampleTable=sampleTable,directory=directory,des
ign= ~time)
```

Sample files for DESeq2 analysis were captured using the “grep” command; this was achieved based on the string match “count”. The file name, treatment condition, and time point of the samplefiles captured in the previous step were set using samplename, samplecondition and the sampletime variables, respectively. These were combined into dataframe (SampleTable) which then formed the input object for DESeqDataSetFromHTseqCount function. DESeqDataSet object must have a design formula (used for calculating the log 2fold change and the dispersion) associated with it. This expresses the variable that will be used in DESeq2 analysis. In this analysis, for each treatment, “time” was used as a variable to calculate the differential expression over and above the control which is defined“time 0”.

Differential Expression Analysis

The default reference level for the factors are set based on the alphabetical order. Hence, the reference level for the variable to calculate differential expression was set using the function “relevel”. Differential expression analysis was calculated using the command “dds”. The contrast argument of the results function was used to extract the log2 fold change between time points. For this purpose the name representing the variable (time), the name of the

factor level for the numerator of the log2 ratio, and the name of the factor level for the denominator were used as options for the contrast argument. A plain text file containing the log2 fold change, *p* value and p_{adj} were exported using base *R* function “write.csv”

```
dds <- DESeq(ddsHTSeq)
dds$time <- relevel(dds$time, "0")
dds <- DESeq(dds)
resultsNames(dds)
```

Extracting Results

Time 0 vs 6hpi

```
res0vs6 <- results(dds, contrast=c("time", "06", "0"))
head(res0vs6[order(res0vs6$pvalue), ], 4)
write.csv(as.data.frame(res0vs6), file="0NIvs6Pba.csv")
```

Transformation

Regularised logarithmic transformation as implemented in the “rlog” function was used to transform the count data, to stabilise the variance across the mean. Rlog transformation of genes with a high count provides similar results to the ordinary log2 transformation of normalised counts. However, for the genes with lower counts, values are shrunken towards the genes’ average across all samples (Love et al., 2014). The following script was used to calculate rlog transformation.

Regularised log transformation

```
rld <- rlogTransformation(dds, blind=FALSE)
rld <- rlog(dds)
head(assay(rld))
rlog <- assay(rld)
rlogMat <- assay(rld)
```

Principal components analysis plot

Principal components analysis (PCA) is a statistical tool that can be used for data quality assessment and sample clustering. In RNA-seq analysis, PCA uses the linear combination of original data, gene expression values or rlog transformed values to define a new set of unrelated variables. Since the similarities and the variance between the data sets are associated with the distance in the projection in space defined by the principal components, a PCA plot is used to observe the overall effect of experimental conditions and the batch effects on the replicates. The following script was used to obtain PCA plot.

```
plotPCA(rld, intgroup=c("condition","time")))
```

2.2.7.3.2 Cuffdiff2

Cuffdiff2 uses an algorithm that controls for the variability across replicate libraries and estimates differential expression at transcript-level resolution (Trapnell et al., 2012). In addition to identifying DEGs and transcripts, Cuffdiff2 also reveals differential splicing, differential expression among isoforms and promoter preference changes (Trapnell et al., 2012). It includes a number of other tools that work together including Cuffmerge, Cuffcompare and Cuffdiff. The Cufflinks tool is the first step in the analysis, where the transcriptome from the RNA-seq data is assembled, and the expression is quantified using FPKM (Fragment Per Kilobase of transcript per Million fragments mapped). Following

assembly, the transcriptomes from the different RNA-seq libraries are compared using cuffcompare. The quality of the assembly is also assessed using cuffcompare. Cuffmerge is used to merge the assemblies from multiple RNA-seq libraries into a master transcriptome. Finally, Cuffdiff is used to identify the gene level differential expression between two different conditions using pairwise comparisons. For this purpose, RNA-seq libraries from each treatment (including all time points) are compared with the non-inoculated control (NI). As in DESeq2, pairwise comparison between the non-inoculated control and treatment are carried out, and the log2 fold change ≥ ±2 and $p_{adj} \leq 0.05$ are used as a cut-off to identify DEGs. The scripts and the options used for the analysis are described below.

Cufflinks

Options:-

-o: All output files created by Cufflinks will have sample name as the prefix

-p: Number of threads to use for Cufflinks

-G: Supply Cufflinks with gene model annotation

```
>cufflinks -o sample_cufflinks.out -p 12
        +-G PGSC_DM_V403_fixed_representative_genes.gff accepted_hits.bam
```

Cuffcompare

Options:-

-r: Annotation (GFF file) is supplied. Each sample is matched against this file, and sample isoforms are tagged as overlapping, matching, or novel where appropriate.

-R: This option causes Cuffcompare to ignore reference transcripts that are not overlapped by any transcript in the RNA-seq sample.

```
cuffcompare -i transcript.lst -r PGSC_DM_V403_fixed_representative_genes.gff -R
```

Cuffmerge

Options:-

-o: Writes the summary stats into a text output file in the specified directory

-g:- Reference annotation file (GTF/GFF). The input assemblies are merged with the reference annotation and included in the final output

-s:- This argument should point to the genomic DNA sequences of the reference genome or a directory containing the one fasta file per contig.

-p:- Number of threads to use

```
>cuffmerge -o ./newMerged.Pecto2015
        + -g PGSC_DM_V403_fixed_representative_genes.gff
         +-s PGSC_DM_v4.03_pseudomolecules_ALL.fasta -p 8 transcript.lst
```

Cuffdiff

Options:-

-p:- Number of threads to use for Cuffdiff

-o:- Sets the name of the output directory where the Cuffdiff outputs are saved

-u:- Performs an initial estimation procedure to more accurately weight reads mapping to multiple locations in the genome

-L:- Label for each sample to be included in the output file

-no-update-check: Turns off the contacts with Cufflinks server to check for a more recent version

```
>cuffdiff -p 12 -o cuffdiff.out -u -L control,sample1,sample2,sample3
        + -no-update-check ../../22.cuffmerge/newMerged.Pect2015/merged.gtf
        +1control.tophat.sorted.bam,2control.tophat.sorted.bam,3control.tophat.sort
        ed.bam
        +1sample1.tophat.sorted.bam,2sample1.tophat.sorted.bam,3sample1.tophat.
        sorted.bam
        +1sample2.tophat.sorted.bam,2sample2.tophat.sorted.bam,3sample2.tophat.
        sorted.bam
        +1sample3.tophat.sorted.bam,2sample3.tophat.sorted.bam,3sample3.tophat.
        sorted.bam
```

2.2.7.4 Alignment of sequence reads to the genomes of SRE

To verify bacterial RNA contamination, the filtered reads were mapped to the reference genomes of *Pba* SCRI1043 (BX950851) (Bell et al., 2004) and *Pbr* ICMP19477 (ALIU00000000) (Panda et al., 2015). Bowtie2-build command was used to build the Bowtie2 index, which was subsequently used to align the reads to the reference.

The following options were used for effective alignment.

--bowtie2--very-sensitive:- bowtie2 option for alignment (default is end-to-end mode)

-S:- Filename for output SAM alignments

-x:- Base name of the index for the reference genome

-p:- Number of threads to align (default is 1)

The below mentioned script was used to build an index and align the reads to the reference genome.

Build Bowtie2-index

```
for x in NZEC1_f.fa SCRI1043_BX950851.1.fasta;
do
bowtie2-build $x $x;
```

Align to Pbr ICMP19477 and Pba SCRI1043 reference genomes

```
>bowtie2 --very-sensitive-local -x NZEC1_f.fa -p 12 -1 sample_R1.fastq
        +-2 sample_R2.fastq -S sample.sam

>bowtie2 --very-sensitive-local -x SCRI1043_BX950851.1.fasta -p 12 -1 sample_R1.fastq
        +-2 sampke_R2.fastq -S sample.sam
```

2.2.8 Functional analysis

The DEGs obtained from DESeq2 analysis were analysed using BLAST2GO (Conesa & Gotz, 2008; Conesa et al., 2005). Translated nucleotide sequences for DEGs were obtained from the Potato Genome Sequencing Consortium (Potato Genome Sequencing Consortium, 2011). The predicted proteome for DEGs was run in BLAST (Altschul et al., 1990), using BLAST2GO (Settings: blastx, nr Database, E value: 1.0E-3, 20 blast hits). Interpro scan (Jones et al., 2014) and Gene Ontology (GO) (Ashburner et al., 2000) analysis were performed to obtain further functional details, in particular for genes with no blast hits. Interpro scan searches multiple databases to look for protein motifs including, but not limited to, TMMHMM, HMM, Blastprodom, Coils. Gene3D, Phobius and SignalP (Conesa et al., 2005). Multilevel GO graphs for the DEGs were obtained using the BLAST2GO combined graph function (Settings: Filter mode Sequence count, Filter value 1). The DEGs were analysed using MapMan to further link predicted functionality with biochemical pathways (Thimm et al., 2004).

2.3 Phenolics

To support the transcriptome data from RNA-seq analysis, the total phenolics in tuber tissue material were assessed using a microplate assay and the phenolics were characterised using liquid chromatography-mass spectroscopy (LC-MS) analysis.

2.3.1 Microplate plate assay

Freeze dried tuber material prepared for the RNA-seq experiment was used in this assay. Samples were prepared by extracting 20 mg of freeze dried tuber material in 1 ml of 80% aqueous acetone. The phenolics were extracted by incubating the samples for 4 h in a rotary

incubator at 4°C. Following incubation, the samples were centrifuged for 10 min at 3800 *xg* at 4°C. Supernatant was transferred to a new tube and stored at -20°C before analysis.

For the microplate assay, 10 µl of each sample was transferred into a microplate well. In addition, 1 mg of gallic acid was dissolved in 1 ml of 80% aqueous acetone. Serial dilutions of the gallic acid solution were then used to obtain a standard curve for further analysis. The samples and the standard were diluted in a 25 µl total volume with distilled water. To the samples and standards, 125 µl 0.2M Folin-Ciocalteu (F-C) (Sigma-Aldrich) was added and incubated for 1 min at room temperature. After incubation, 100 µl of 7.5% (w/v) Na_2CO_3 was added to the reaction mixture. Colour development due to the reaction of phenolics with the Phosphomolybdic-phosphotungstic acid in the F-C reagent was measured at 756 nm using a microplate reader at 45°C for 15 min. Samples were run in triplicate.

2.3.2 Liquid chromatography-mass spectroscopy

Samples for LC-MS analysis were prepared by extracting 10 mg of freeze dried tuber material (prepared for RNA-seq experiment) in 1 ml of 1:1 methanol and water. LC-MS analysis was carried out by Nigel Joyce (Senior Research Technologist, Plant and Food Research)

The LC-MS system consisted of a Thermo Electron Corporation (San Jose, CA, USA) Accela UHPLC pump, Thermo Accela Open Auto sampler (PAL HTC-xt with DLW), Finnigan Surveyor PDA plus detector and a ThermaSphere TS-130 column heater (Phenomenex, USA). Each of the 48 extracts was analysed by two ion formation modes creating 96 data files.

A 5 µL aliquot of each prepared extract was separated with a mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) by reverse phase chromatography (Kinetex guard cartridge and Kinetex C18, 2.6 µ, 100 Å, 100 x 2.1 mm, Phenomenex, USA), maintained at 30°C with a flow rate of 250 µl/min. A gradient was applied as follows: 0-2 min/98%A, 10 min/50%A, 11-16min/0%A, 19-25min/98%A.

The eluent was scanned by API-MS (LTQ, 2D linear ion-trap, Thermo-Finnigan, San Jose, CA, USA) with electrospray ionisation (ESI) in the negative and positive mode. Data were acquired for precursor masses from m/z 110–1500 amu with up to MS3 product spectral tree formation.

Data were processed with the aid of Xcalibur®2.20 (Thermo Electron Corporation) and Mass Frontier™ 7 SR1 (Thermo Electron Corporation), XCMS online (https://xcmsonline.scripps.edu/), Lipid Maps (http://www.lipidmaps.org/), Mass Bank (http://www.massbank.jp) and an in-house PFR database.

2.4 CFA expression and purification

2.4.1 Preparation of RNA samples to quantify transcription of *cfa* genes in *Pectobacterium* spp.

To compare the transcription of the *cfa* biosynthetic genes in tubers, stems and *in vitro* cultures, potato ('Summer Delight') tissues were inoculated with *Pba* SCRI1043 as described in section 2.1.1.1 and 2.1.1.2, or bacteria were grown in MM+M9 (section 2.1.1) at 28°C. Bacterial cells were isolated from diseased potato stems at 10 dpi, from inoculated tuber tissue at 7 dpi or from liquid cultures at 48 hpi (when the cultures were in stationary phase). Total RNA was then extracted from bacterial cells using the Promega SV total RNA kit as described in section 2.4.2.

2.4.2 Isolation of total RNA

2.4.2.1 Isolation of bacterial RNA from *in vitro* cultures

Bacterial cells were cultured as described in section 2.1.1. Total RNA was then extracted by harvesting cells by centrifugation for 8 min at 16000 *g*. The bacterial pellet was resuspended in 100 µl TE buffer containing at least 1 mg/ml lysozyme. The mixture was incubated at room temperature for 5 min. Total RNA from the harvested cells was isolated using the Promega SV total RNA isolation kit. To the sample, 175 µl lysis buffer was added, and the resulting sample was mixed well by inversion. After this, 350 µl RNA dilution buffer was added. The sample was then centrifuged for 10 min at 16000 *g*. The resulting supernatant was transferred into a clean tubes containing 1 ml absolute ethanol and mixed well. Each sample was then incubated at -80°C for 60 min. Following incubation, each sample was centrifuged at 16000 *g* in 4°C for 5 min; the pellet was washed with 600 µl wash buffer, and the supernatant was discarded. The resulting RNA was air dried and resuspended in RNase-free water. For on-column DNase treatment, 2 volumes of absolute ethanol were added to the RNA samples. The mixture was then transferred into a spin column (Promega SV total RNA isolation kit) and centrifuged for 30 s at 16000 *g*. The elute was discarded, and the column was washed with the wash buffer. RNA bound to the column was subjected to DNase treatment as described in the manufacturer's protocol. Extracted total RNA was stored at -80°C.

2.4.2.2 Isolation of total RNA from inoculated potato tubers

Total RNA from infected potato tubers was isolated as described by Liu et al (Liu et al., 2008). Briefly, at 7 dpi, cells were isolated from the tubers by scraping the tissues around the inoculation site into SDW. Starch from the potato tubers was removed by centrifugation twice at 100 *g* for 1 min. The bacterial cells in the supernatant were transferred into RNA stabilisation buffer (pH 4.3, v/v), containing 1% phenol and 20% absolute ethanol (v/v), followed by incubation on ice for at least 30 min. Total RNA was isolated using Promega SV

total RNA isolation kit, including DNase I treatment, as described in the manufacturer's protocol. Extracted total RNA was subsequently stored at -80°C.

2.4.2.3 Isolation of total RNA from inoculated potato stems

Total RNA from infected plant stem was isolated using the Promega SV total RNA isolation kit. The entire lesion from each infected potato stem was harvested at 10 dpi and was ground to fine powder in liquid nitrogen using a mortar and pestle and stored at -80°C. Approximately 300 mg of powdered plant material was then placed in a frozen 15 ml centrifuge tube and was further ground in 1 ml of lysis buffer containing β-mercaptoethanol using a RNase-free polypropylene pestle. Subsequently, total RNA was isolated and subjected to DNase treatment as described in the manufacturer's protocol. Extracted total RNA was stored at -80°C.

2.4.3 Isolation of bacterial DNA from *in vitro* cultures

Bacterial cells were cultured in LB as described in section 2.1.1. Bacterial cells were harvested by centrifugation for 8 min at 16000 *g*. Genomic DNA was isolated using the DNeasy blood and tissue kit (Qiagen). Isolation was performed as recommended by the kit's manufacturer and DNA was eluted in 70 µL of elution buffer (EB) by centrifugation of the spin column at 5900 *g* for 1 min.

2.4.4 Measurement of transcription of *cfa* genes in *Pba* SCRI1043 using qRT-PCR

cDNA was synthesised from total RNA samples using a SuperScript VILO cDNA synthesis kit (Invitrogen). The Superscript enzyme mix included the SuperScript III RT and RNaseOUT recombinant ribonuclease inhibitor. The 5X VILO reaction mix contained the random primers,

$MgCl_2$ and dNTPs. Reactions were carried out in a 20 µl volume containing 4 µl of 5X VILO reaction mix, 2 µl 10X SuperScript enzyme mix, 2 µg total RNA and nuclease-free water to bring the volume to 20 µl. The reaction mix was incubated at 25°C for 10 min followed by 42°C for 60 min. The reaction was terminated by incubation at 85°C for 5 min.

Sybr Green based qRT-PCR was used to quantify the relative expression of *cfa* genes under different conditions. The Applied Biosystems StepOne Plus™ was used for real time fluorescence detection of PCR products and the results were analysed with Applied Biosystems StepOne software V2.1. For each qRT-PCR reaction, a 1 in 5 dilution of prepared cDNA was used as a template. Reactions were carried out in 11 µl, containing 5.5 µl of Sybr Green master mix, a final concentration of 300 nM of each primer and nuclease-free water to bring the volume up to 10 µl and 1 µl of cDNA.

Primers (Table 2.6) were designed for amplification of the *cfa6* and *cfa7* genes using Geneious Pro R6 (http://www.geneious.com). The genes encode the two polyketide synthases required for the biosynthesis of CFA. For each gene, primer concentrations for qRT-PCR analysis were optimised by performing reactions with each primer pair for each gene at four different concentrations (100 nM, 200 nM, 300 nM and 400 nM). The optimal annealing temperature for each primer pair was then determined using temperature gradient qRT-PCR. The optimised primer concentration was 300 nM (Table 2.6), and the annealing temperature was 60°C for each target sequence. Amplification reactions were performed in quadruplicate. The cycling conditions were 95°C for 3 min; followed by 40 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 20 s, with data capture during the extension phase of each cycle. Melt curve analysis was undertaken for all reactions to confirm amplification of the appropriate product.

To estimate the relative expression levels of target genes, calibration standards were developed using a series of 10-fold serial dilutions of genomic DNA isolated from *Pba* SCRI1043 (starting concentration of 100 ng per µl). The target genes were normalised against the *ffh* gene (encoding signal recognition particle protein). The *ffh* gene has stable expression under different culture conditions as well as *in planta* (Takle et al., 2007). To determine the relative expression level of the genes, linear regression of C_T values (cycle threshold) for the standards on the $\log_{10}$ Copy Number (CN) were carried out using equation 1 (below). The

efficiency factor (Eff) was calculated using equation 2. Calibration parameters were used to estimate DNA quantities/ dilutions from C_T values for samples using Equation 3.

Equation 1: $C_T = Const + Slope \times \log_{10} CN$

Equation 2: $Eff = 10^{(-1 / Slope)}$

Equation 3: $Dilution = 10^{(CT-Const)/ Slope}$

Before further analysis, the mean C_T of the four replicates for each sample was calculated. The mean C_T for each sample was converted into the ratio of the quantity of the test gene to the quantity of the reference gene (*ffh*) using the estimated calibration parameters (described in the above equations). The $\log_{10}$ of these ratios was analysed with analysis of variance. All analyses were carried out with GenStat (GenStat Committee, 2011).

Table 2.6: Primers used in this study.

Gene Name	Primer Name	Primer concentration (nM)	Primer sequence (5′ to 3′)
ffh	ffh-F	300	ATGGGCGATGTGCTTTCACT
	ffh-R	300	TCAAACCCATCGCCTTTCTT
cfa6	cfa6-F	300	GGGTTCTCGTTCTGTGCTGA
	cfa6-R	300	TGATCACACACCATGCGACA
cfa7	cfa7-F	300	AGATTGAATACGCCGGCGAT
	cfa7-R	300	GTGCCGAACTGTCATGCATC

2.4.5 Detection of CFA using GC-MS

For gas chromatography-mass spectroscopy (GC-MS), carboxylic acids were extracted from approximately 200 mg of freeze dried samples (either *in vitro* culture filtrate or freeze dried plant material). Carboxylic acids were separated from the largely non-volatile mix by vapour phase extraction (VPE) (Mishina & Zeier, 2006; Schmelz et al., 2004). Metabolite extraction was performed by adding 600 µl of 1-propanol: water: HCl (2:1:0.005) to the sample and

vortexing for 30 s. As an internal standard, 20 µl of jasmonic acid stock solution (10 µg/ml) and 1 ml of dichloromethane ($MeCl_2$) were added to each sample, vortexed for 5 s and subjected to centrifugation at 11300 *g* for 30 s. The lower organic phase ($MeCl_2$:1-propanol) layer was removed, transferred into a 4 ml glass vial and dried over sodium sulphate. The carboxylic acids in the $MeCl_2$:1-propanol layer were converted to methyl esters by adding 2 µl of a 2 M solution of trimethylsilyldiazomethane in hexane. The vials were vortexed and incubated at room temperature for 5 min. Methylation reactions were stopped by adding 2 µl of 2 M acetic acid. This increases the volatility of carboxylic acids and jasmonic acid (internal standard) and enabled separation by gas chromatography. Analytes were trapped on Super Q absorbent (Alltech, USA), a highly stable divinylbenzene polymer tolerant to H_2O vapour and sensitive only to temperatures above 300°C. This method involved two evaporation steps, first at 70 C and then at 200°C, to make use of the Super Q adsorbent properties and increase the range of analytes recovered. The 4 ml vials were closed with a high temperature septum (Schott, Germany), and volatile collection traps containing 30 mg Super Q (ARS, USA) were inserted through the septum along with a needle that supplied a gentle stream of nitrogen (flow rate 0.8 l min^{-1}) (Figure 2.3). The connected vial was placed in a dry block heater adjusted to 70°C to expedite the evaporation of the derivatised extract. When the solvent was evaporated (2-3 min), the vial was transferred to a second heating block at 200°C for 2 min. This step was required to collect compounds of lower volatility. Once the VPE was completed, all the samples were eluted from the filters with 1 ml dichloromethane into reaction vials. The sample volume was finally reduced to about 50 µl under a stream of N_2 and samples were stored at -80°C or immediately analysed by GS-MS. Super Q traps were rinsed with 300 µl of dichloromethane before each reuse. GC-MS analysis was carried out by Jason Breitmeyer (Lincoln University, New Zealand).

Figure 2.3: Diagrammatic representation of the layout used for metabolite extraction. The spatial arrangement of the super Q filter (A), N_2 inlet stream during VPE and general layout of VPE apparatus (B)(Schmelz et al., 2004).

Samples were analysed using a Shimadzu GCMS-QP2010 Ultra (Shimadzu, Japan) gas chromatograph mass spectrometer fitted with a Restek Rtx-5ms fused silica capillary column (30.0 m x 0.25 mm i.d. x 0.25 µm, Bellefonte, PA, USA). A CTC-Combi PAL autosampler (Shimadzu AOC-5000) was used to inject 1 µL of sample into the GC injection port, operating in high pressure injection splitless mode at 220 °C and 241 kPa for 40 s. After injection, the column oven was held at 50°C for 3 min, then heated to 320°C at 8°C min^{-1}, and held at this temperature for 8 min. Helium was used as the carrier gas with constant linear velocity set at 44.4 cm/s in split mode (1.5 ml min^{-1}). The mass spectrometer was operated in single ion monitoring mode with selected masses used to identify CFA (target ion m/z 222; confirming ions m/z 190 and m/z 151), and CFA-valine (target ion m/z 191, m/z 321; confirming ion m/z 190) and internal

standard methyl jasmonate (target ion m/z 151, confirming ions–m/z 193 and m/z 224). The temperature of the capillary interface was 320°C, with the MS source temperature set at 230°C.

Initial confirmation of retention times was performed for the compound of interest by injecting the exogenous CFA standard (10 µg/ml) (kindly provided by Robin Mitchel and David Greenwood Plant and Food Research, New Zealand) and confirming the mass spectra with that expected for the molecule. The software used to acquire and process the data files was GCMS solution version 2.72 (Shimadzu, Japan).

Chapter 3 A comparison of two software packages for analysing the transcriptional response of potato to *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* subsp. *brasiliensis*

3.1 Summary

Total RNA-seq has proven to be useful in revealing the unprecedented complexity of plant-microbe interactions. To enhance our understanding of the molecular interactions between potato and pectobacteria, this study used Illumina HiSeq to measure DE in potato tubers ('Summer Delight') during the initial stages of a susceptible interaction with *Pba* SCRI1043 and *Pbr* ICMP19477, and examined the immune responses of the host to these pathogens. In addition, the effect of CFA, an important phytotoxin for both SRE, was studied by RNA-seq of potato tubers either inoculated with a *Pba* SCRI1043 mutant in which the CFA biosynthetic cluster had been removed or upon exposure to exogenous CFA. A total of, 48 RNA samples from inoculated potato tubers were isolated during the early (6 hpi), intermediate (12 hpi) and later stages (24 hpi) of disease progression, as determined by analysis of bacterial population growth in tubers after inoculation, or after application of exogenous CFA. These samples generated 2.3 billion reads, with an average of ~47 Million reads per sample. Alignment of the reads to the potato reference genome PGSC v4.03 mapped ~70% of the reads to the host. The DEGs were subsequently identified using the mapped reads from the different treatments using the two commonly available software packages DESeq2 and Cuffdiff2. A comparative analysis of the DEGs identified using these software packages showed that the former was the more robust method for differential gene expression analysis. In contrast, Cuffdiff2 consistently generated higher numbers of false positives as determined by the read count analysis. On the basis of these findings, the differential expression data generated by DESeq2 was chosen for use in subsequent chapters to identify key pathways in the immune response of potato to *Pba* and *Pbr*.

3.2 Background

The main aim of transcriptomics is to characterise all species of RNA including mRNA, non-coding RNA and small RNAs, to determine the structure of each gene, and to quantify the changing expression level of the gene's transcription under different conditions (Tang et al., 2009). Thus, transcriptomics has enabled deeper understanding of the changes in gene expression that occur in organisms in various biologically important situations, such as when a plant is invaded by a pathogen (Faino et al., 2012; Howard et al., 2013; Yogendra & Kushalappa, 2016). Knowledge of these changes has enabled a greater understanding of the methods used by pathogens to colonise their plant host and the responses plants' have evolved to counteract invasion.

RNA-seq, a recently developed technology that applies the principles of Next Generation Sequencing (NGS) to cDNAs obtained from the total RNA populations in a sample, has become the primary choice for studying the transcriptional changes that occur during interactions between two organisms. The fundamental principle of RNA-seq is that the rate at which a certain RNA molecule in a given RNA mixture is sequenced is proportional to the number of copies of this RNA molecule in the mix (Ozsolak & Milos, 2011). The abundance of the sequence obtained using this technique provides several advantages compared to microarray technologies, including the ability to detect novel transcripts and transcript isoforms, the capacity to map transcripts to the genome and enabling quantification of transcripts. In this study, Illumina HiSeq, one of the predominant NGS platforms currently in use, was used to examine the immune response of potato during the initial stages of a susceptible interaction with the SRE pathogens *Pba* SCRI1043 and *Pbr* ICMP19477. It was anticipated that an understanding of the mechanisms underlying the transcriptional response of potato to these related pathogens might provide opportunities to combat the economically damaging diseases they cause.

Pectobacterium spp. are the main causative agents of aerial stem rot and blackleg in potato during crop growth and soft rot of tubers during storage (Perombelon, 2002; Perombelon & Kelman, 1980). Amongst the myriad of *Pectobacterium* spp. and subspecies, *Pba* and *Pcc* are

the two primary taxa that are isolated from infected potato tubers and plants in New Zealand (Crowhurst & Wright, 1988; Pitman et al., 2008). Indeed, *Pba* has been considered for some time to be the main seed borne enterobacterial pathogen responsible for blackleg (Czajkowski et al., 2015; Moleleki et al., 2013). *Pbr* has recently become more prevalent, however, with new reports suggesting it has superseded *Pba* and *Dickeya* spp. as the predominant cause of blackleg in Dutch seed potatoes (*New bacteria main cause of blackleg in Dutch seed potatoes*, 2016). Numerous studies that have compared the aggressiveness of *Pectobacterium* spp. on potato have identified *Pbr* as far more aggressive than many of the other soft rotting and blackleg causing enterobacteria (Duarte et al., 2004; Marquez-Villavicencio et al., 2011; Panda et al., 2012; Pitman et al., 2008; van der Merwe et al., 2010).

Comparative genome analysis of several SREs, including *Pba* and *Pbr*, have confirmed that these taxa share a common, core genome (representing approximately 50% of the nucleotides from each species), but also possess variable accessory genomes that likely contribute to niche differentiation or differences in aggressiveness (Glasner et al., 2008; Panda, 2014). Indeed, much of their accessory genomes are comprised of HAIs and gene islets, unique mobile genetic elements in each species that carry genes such as antimicrobial biosynthesis genes, PCWDEs, sugar metabolism genes and phytotoxin biosynthetic clusters (Bell et al., 2004). A phytotoxin biosynthetic cluster on one of the HAIs in *Pba* and *Pbr* encodes CFA (Bell et al., 2004; Panda, 2014; Panda et al., 2016). As described in Chapter 1, CFA is important for virulence in SRE, inoculation of susceptible plants with a *Pba* strain having a deletion of one of the two polyketide synthases (*cfa6* and *cfa7*) resulting in reduced symptoms of blackleg (Bell et al., 2004). Furthermore, *Pba* SCRI1043ΔHAI2, a strain in which the entire HAI was removed from the genome by CRISPR-Cas-mediated genome targeting, also causes reduced blackleg symptoms compared to the wild type when inoculated into potato stems (Panda et al., 2016; Richter & Fineran, 2013; Vercoe et al., 2013). CFA is a mimic of methyl jasmonate and has been shown to induce a genetic response in tomato if applied exogenously (Uppalapati et al., 2005).

Though much is known about the mechanisms that *Pectobacterium* spp. use to colonise potato and to elicit a disease response, no transcriptomics studies have been undertaken to examine the immune responses of the host or to examine how specific virulence factors might

influence the molecular processes involved in these responses. This study used Illumina HiSeq to examine the immune responses of the host during the initial stages of a susceptible interaction with *Pba* SCRI1043 and *Pbr* ICMP19477, and to investigate the effect of CFA on this interaction. This was done by extracting total RNA samples from potato tubers exposed to either wild type pathogens, a *Pba* mutant in which the CFA biosynthetic cluster had been removed or exogenous CFA. As *Pectobacterium* spp. usually colonise potato through lenticels and wounds in the potato tuber or through the roots (Perombelon, 1992, 2002; Perombelon & Hyman, 1989; Perombelon & Kelman, 1980), it was assumed that studying the response in tubers would demonstrate the first transcriptional responses undertaken by the plant upon invasion by the pathogen.

Whilst undertaking this transcriptome study, alternative software for the analysis of transcript abundance were tested. Previously, in my MSc study, Cuffdiff (Trapnell et al., 2010) was used to analyse differential expression from Illumina HiSeq data (Ramakrishnan, 2012). However, concurrent with the increasing popularity of RNA-seq, a variety of computational software packages have been introduced for differential expression analysis, including DESeq2, edgeR, SAMseq, limma, Cuffdiff2 to name a few. Selection of data analysis approach depends on the experimental design, the number of replicates (both technical and biological replicates) and the objective of the study. Due to the lack of consensus over a pipeline for differential expression analysis and the variation in results from different tools, in this study two well documented computational software tools, DESeq2 (Love et al., 2013) and Cuffdiff2 (Trapnell et al., 2013) were used to identify differential expression in potato. Their suitability for functional studies in subsequent chapters was then compared.

3.3 Results

3.3.1 The impact of *Pectobacterium* spp. and CFA on potato tubers

3.3.1.1 The aggressiveness of *Pectobacterium* spp. and a CFA mutant on potato tubers

To confirm the aggressiveness of different *Pectobacterium* spp. on potato, eight tubers ('Summer Delight') were inoculated with either *Pba* SCRI1043 or *Pbr* ICMP19477 (Section 2.2.1; Table 3.1). Tubers were also inoculated with *Pba* SCRI1043ΔHAI2 (Table 3.1) to establish the impact that deletion of HAI2 (and consequently the CFA cluster) has on the virulence of *Pba*. The average percentages of tuber weight loss were then measured 7 dpi, which showed substantial variation between strains ($p < 0.001$). Indeed, average percentage tuber weight loss caused by *Pbr* ICMP19477 was 8.87%, significantly higher ($p < 0.05$) than that caused by *Pba* SCRI1043 (4.01%) (Table 3.1). When compared to *Pba* SCRI1043, however, no significant difference in weight loss was observed in tubers treated with *Pba* SCRI1043ΔHAI2, suggesting that HAI2 (and CFA) may not be required by the pathogen during soft rot disease progression on this cultivar or that CFA was responsible for only very subtle physiological or molecular changes in the host.

Table 3.1: Mean percentage weight loss observed in potato tubers ('Summer Delight') 7 dpi with various strains of *Pectobacterium*.

Strain	Weight loss
Pba SCRI1043	4.01 (2.82,5.68)
Pba SCRI1043ΔHAI2	3.41 (2.35,4.94)
Pbr ICMP19477	8.87 (7.11,11.00)
Control	0.26 (0.01,5.26)

Note: The numbers in parenthesis show the 95% confidence limit

3.3.1.2 Population growth of *Pectobacterium* spp. in potato tubers

Having demonstrated the aggressiveness of *Pba* SCRI1043, *Pbr* ICMP19477 and *Pba* SCRI1043ΔHAI2 on potato tubers, suitable time points were identified to extract total RNA from tubers in order to study the early transcriptional response of the host using RNA-seq analysis. This was achieved by analysing the growth dynamics of each strain upon infection. Specifically, 15 tubers of 'Summer Delight' were inoculated with either *Pba* SCRI1043, *Pbr* ICMP19477 or *Pba* SCRI1043ΔHAI2 and the populations of each bacteria at the point of inoculation were sampled at 0, 12 hpi, 1, 2, 5 and 6 dpi as described in section 2.2.3.

Though the overall CFU estimates from each tuber were relatively similar, subtle variability was observed between the replicates of the same strain and between sampling times. Thus, the pattern of growth as estimated by mean CFUs over time varied between *Pba* SCRI1043, *Pba* SCRI1043ΔHAI2 and *Pbr* ICMP19477 (p = 0.013 for the strain by time interaction). More specifically, mean CFUs of *Pbr* ICMP19477 were marginally different from *Pba* SCRI1043 and *Pba* SCRI1043ΔHAI2, particularly with lower CFUs for 12 hpi and 1 dpi and higher CFUs for 3 and 7 dpi. The estimated mean CFUs for *Pba* SCRI1043ΔHAI2 were very similar to those for *Pba* SCRI1043 (Figure 3.1).

As a result of these experiments, it was decided to extract total RNA at 6, 12 and 24 hpi, which coincided with the late lag to early log phase, early to mid-log phase, and late-log to early stationary phase for all strains. These phases were considered to represent the earliest phases of the interaction between the host and the pathogen, prior to the pathogen completely overcoming the plant and eliciting disease. In addition, at these time points there were very few differences in CFU counts for different strains, suggesting any transcriptional differences in the host would be due to specific responses to the different strains and not due to the effect of the pathogens being at different phases in their growth.

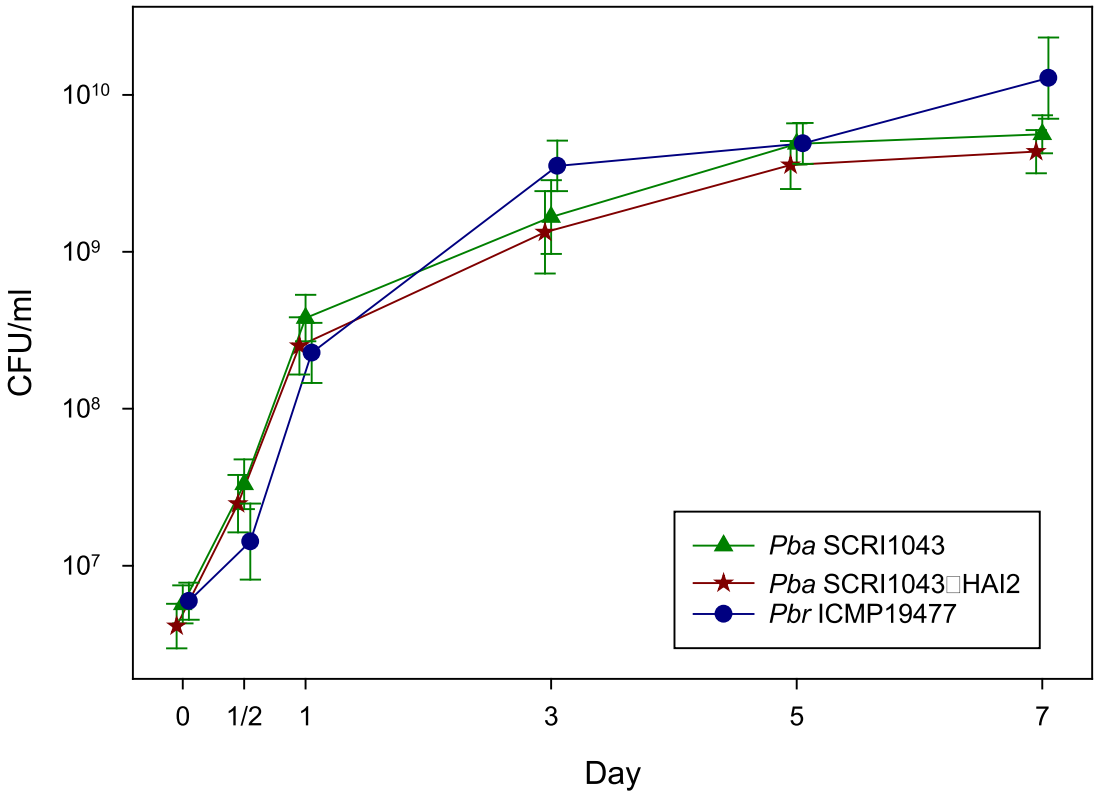


Figure 3.1: Estimated mean CFU/ml at each assessment for tubers ('Summer Delight') treated with either *Pba* SCRI1043, *Pba* SCRI1043ΔHAI2 or *Pbr* ICMP19477.
Error bars are 95% confidence limits.

3.3.1.3 Symptomology in potato tubers associated with application of exogenous CFA

Pba SCRI1043ΔHAI2 showed no visible difference in maceration of potato tubers when compared to the wild-type strain. The fact that the mutant had shown reduced symptoms in the stem (Panda et al., 2016) and that CFA had previously been shown to alter host transcription in *Arabidopsis* and tomato (Mittal & Davis, 1995; Uppalapati et al., 2005), however, suggested that CFA might manipulate the host defence response in potato tubers at the cellular level without resulting in dramatic changes in disease symptoms. Hence, in addition to studying the transcriptional responses of *Pba* SCRI1043 and *Pbr* ICMP19477, the effect of *Pba* SCRI1043ΔHAI2 and application of exogenous CFA was examined using RNA-seq. Previous research on tomato applied 0.2-20 nM (Uppalapati et al., 2005) of CFA to

seedling/leaves to examine the physiological/transcriptional response, although symptoms were not observed at the concentration applied. To identify whether higher concentrations of CFA elicited disease symptoms in potato and whether a particular concentration was required to study the transcriptional response, potato tubers ('Summer Delight') were inoculated with exogenous CFA at concentrations of between 20 and 200 nM (section 2.2.2). In these experiments, inoculated tubers showed no distinct disease symptoms regardless of dose, although a small amount of necrotic tissue developed at the point of inoculation (Figure 3.2 C & D). In contrast, potato tubers inoculated with *Pbr* ICMP19477 showed severe soft rotting with lesion lengths ranging from 30-40 mm (Figure 3.2 A) and *Pba* SCRI1043 (Figure 3.2 B) produced lesion lengths of 18-20 mm around the point of inoculation (confirming the greater aggressiveness of *Pbr* ICMP19477). The limited necrosis with exposure of the tubers to CFA was consistent with the lack of visible phytotoxicity in previous studies on tomato (Uppalapati et al., 2005). Furthermore, Wasternack et al (1998) reported that a 250 µM concentration of CFA on detached tomato leaves failed to elicit JA-related transcriptional responses. Taken together, the results from this analysis suggested that applying nano molar concentrations of exogenous CFA to tubers would be sufficient to examine genetic changes in the host associated with this phytotoxin even in the absence of symptoms. Thus, transcriptional studies were performed on tubers exposed to 200 nM CFA.

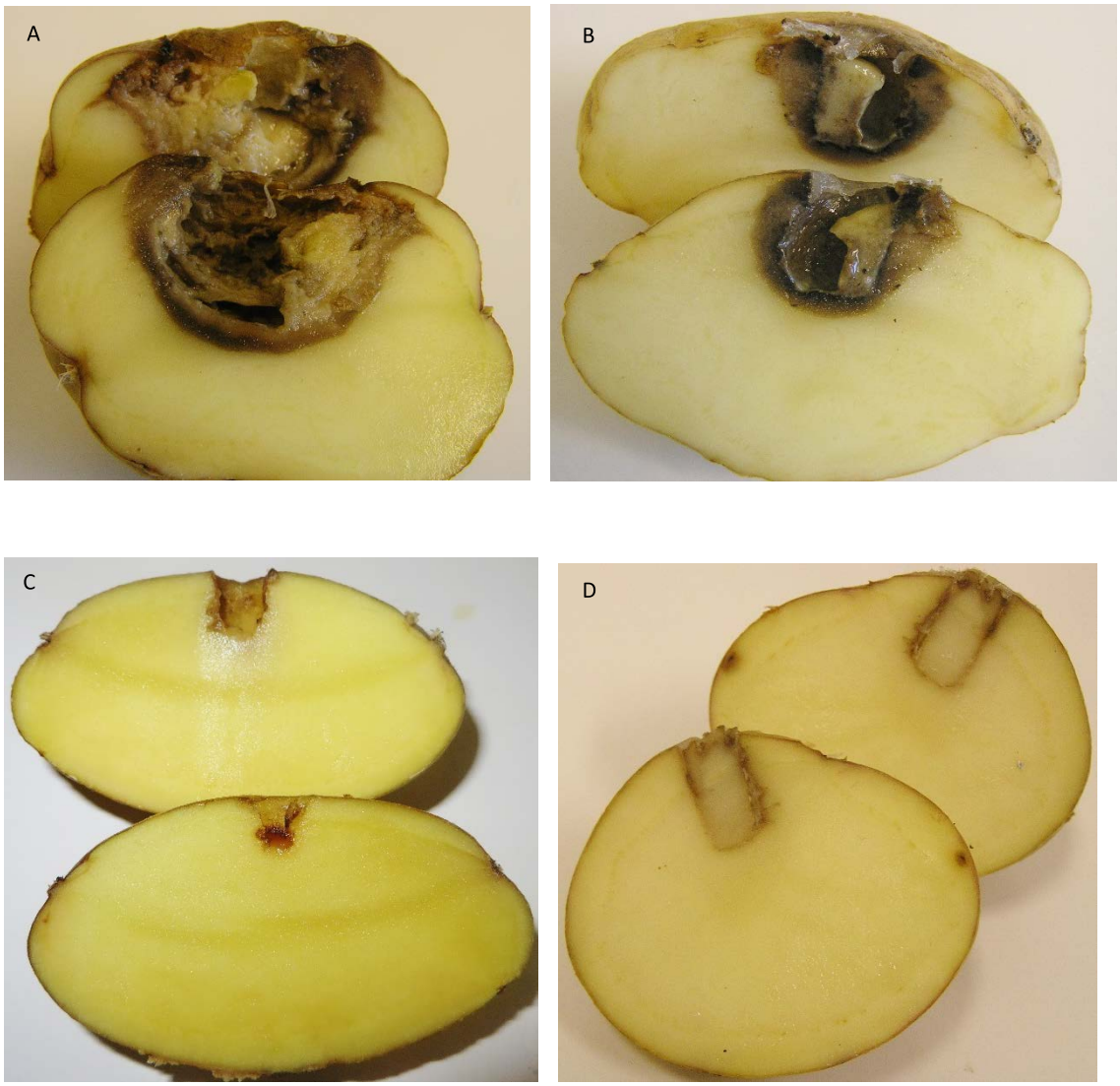


Figure 3.2: Soft rot lesions in potato tubers ('Summer Delight') inoculated with either (A) *Pbr* ICMP19477, (B) *Pba* SCRI1043, (C) Coronafacic acid 20 nM or (D) Coronafacic acid 200 nM at 7 days post inoculation.

3.3.2 Differential expression in potato tubers in response to *Pectobacterium* spp. and exogenous CFA

After establishing the growth dynamics of *Pba* SCRI1043, *Pbr* ICMP19477 and *Pba* SCRI1043ΔHAI2 in potato tubers of 'Summer Delight' and the dose response to exogenous CFA, to examine host transcription during interactions with these bacteria and with the phytotoxin a transcriptome experiment was undertaken as described in section 2.2.4. As a result of this experiment, a total of 48 RNA samples were extracted from tubers exposed to

either of the treatments using the LiCl-based method described in section 2.2.5. RNA-seq was then performed on the libraries prepared from the total RNA using the Illumina HiSeq 2000 platform (NZGL) (section 2.2.6).

3.3.2.1 Total RNA integrity

The average RNA integrity number (RIN) measured using Bioanalyzer (section 2.2.5.2) was ~8.1 with RIN values for all 48 samples being ≥ 7 (Table 3.2). A value of 7 is the minimum value recommended by the sequence providers to ensure robust RNA-seq data are obtained for downstream analysis.

Table 3.2: RIN values of total RNAs extracted from potato tubers ('Summer Delight').

Treatment	Replicate	0 hpi	6 hpi	12 hpi	24 hpi
Pba SCRI1043	1	-	8.5	8.3	9.2
	2	-	8.2	7.6	8.6
	3	-	7.0	8.4	8.2
Pba SCRI1043ΔHAI2	1	-	8.3	7.0	8.2
	2	-	8.6	7.8	8.2
	3	-	9.3	8.1	8.3
Pbr ICMP19477	1	-	8.5	8.3	7.6
	2	-	8.5	8.2	6.7
	3	-	8.5	7.5	7.3
CFA (200 nM)	1	-	8.6	7.8	8.1
	2	-	8.3	8.3	7.4
	3	-	7.8	7.9	7.9
Mock-inoculation (10 mM $MgCl_2$)	1	-	8.6	8.3	8.3
	2	-	8.6	8.5	7.4
	3	-	8.6	8.8	8.2
Non-inoculated control	1	9.0	-	-	-
	2	8.6	-	-	-
	3	8.1	-	-	-

3.3.2.2 Quality control assessment of RNA-seq data

3.3.2.2.1 FastQC for RNA-seq dataset

An average of ~47 Million paired end reads per sample were obtained using the Illumina HiSeq 2000 platform, which were provided in a Fastq format. About 95.5% of the bases had data quality scores ≥ Q30, with a mean quality score of 36. Thus, after the initial quality control (section 2.2.7.1 for the script), an average of 46 Million reads with a read length of at least 86 bp was obtained from each sample.

3.3.2.2.2 Alignment of the RNA-seq reads to the potato reference genome

On average, approximately 70% (Appendix A.1) of the filtered reads were mapped to the potato reference genome (PGSC-v4.3) (Sharma et al., 2013) using the splice aware alignment combination of TopHat (Kim et al., 2013) and Bowtie2 (Langmead & Salzberg, 2012).

3.3.2.2.3 Alignment of RNA-seq read mapping to the genome of pectobacteria

The average percentage of reads mapped to the reference genomes of either *Pba* SCRI1043 or *Pbr* ICMP19477 was 0.2-0.3% (Appendix A.1). As the RNA samples were taken from distal tissue, the low quantity of reads mapping to the pathogen was expected, and demonstrated that RNA samples had not been contaminated by bacterial RNA.

3.3.2.2.4 Principal component analysis of the RNA-seq dataset

RNA-seq reads mapping to the reference genome PGSC-v4.3 in each of the 48 samples were compared using a PCA (section 2.2.8.3). PCA showed that PC1 (time) was the dimension exhibiting the greatest variation (51% of the variance), clearly separating RNA samples collected at different time points (Figure 3.3). In contrast, only 14% of the variance in the experimental dataset was attributed to PC2 (variation between treatments with bacteria or CFA or controls) (Figure 3.3). Together, PC1 and PC2 explained > 60% of the total variance in this dataset.

PCA showed that RNA-seq data obtained from total RNA samples collected at 6, 12 and 24 hpi were distinct. However, the samples from non-inoculated controls were more variable and appeared to overlap with the samples collected from 6 and 12 hpi. In addition, greater within-treatment sample variation was observed at 12 and 24 hpi when compared to the samples at 6 hpi. These results indicate that time plays a significant role in defining the overall transcriptome dynamics.

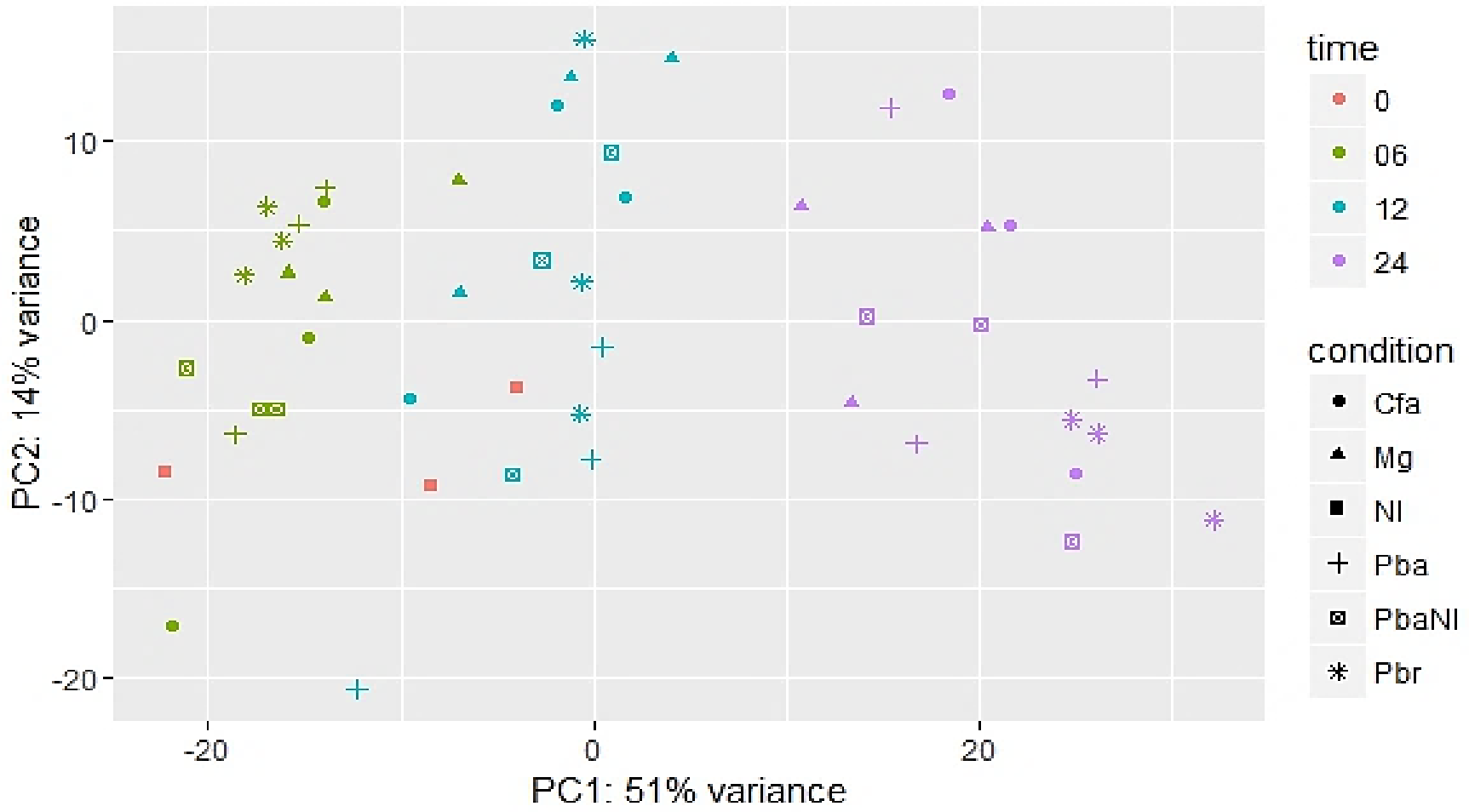


Figure 3.3: PCA plot on rlog transformed data from 48 RNA samples from potato tubers ('Summer Delight') traversed by their first two principal components. Time 0, 06, 12 and 24 represent the time points (in hours) used in RNA-seq and conditions CFA, Mg, NI, Pba, PbaNI and Pbr represent Coronafacic acid (200 nM), mock-inoculation control, non-inoculation control, *Pba* SCRI1043, *Pba* SCRI1043ΔHAI2 and *Pbr* ICMP19477 treatments, respectively.

3.3.2.3 Differential Expression analysis

To identify differential expression in potato tubers treated with *Pectobacterium* spp. or with CFA, pairwise comparisons were conducted using the mapped reads from non-inoculated control tubers (0 hpi) and tubers treated with bacteria or exogenous CFA at each time point. Datasets from mock-inoculated samples were also compared to the non-inoculated controls to identify differential expression resulting from the wounding of the tubers during inoculation with either the bacteria or with exogenous CFA. Pairwise comparisons of untreated and treated samples is the standard approach to differential expression analysis, which tests the null hypothesis (i.e., the observed log fold change for a gene between the control and the treatment is zero, which suggests that the expression of a gene is not altered by the treatment).

Pairwise comparisons were performed using Cuffdiff2 (Trapnell et al., 2013) and DESeq2 (Love et al., 2013). The pipelines for analyses using Cuffdiff2 and DESeq2 are shown in sections 2.2.8.3. Log2 fold change ≥ ±2 and $p_{adj} \leq 0.05$ were used as cut-offs to identify DEGs in both Cuffdiff2 and DESeq2 analyses.

3.3.2.3.1 Differential gene expression analysis using Cuffdiff2

A total of 9130 genes were DE in tubers exposed to either bacteria, CFA or mock-inoculation when compared to the non-inoculated control (at one or more sampling times). In general, the greatest numbers of DEGs were expressed at 24 hpi (Figure 3.4), with a total of 1511, 2411 and 5208 DEGs identified by Cuffdiff2 at 6, 12, and 24 hpi, respectively. For tubers inoculated with bacteria, the number of DEGs was greatest after inoculation with *Pbr* ICMP19477 (a total of 408, 626, and 1559 genes at 6, 12 and 24 hpi, respectively) rather than *Pba* SCRI1043 or *Pba* SCRI1043ΔHAI2. Inoculation with *Pba* SCRI1043 or *Pba* SCRI1043ΔHAI2 resulted in similar numbers of DEGs in potato tubers. The total number of DEGs in response to *Pba* SCRI1043 were 365 (218 up regulated and 147 down regulated), 352 (203 up regulated and 149 down regulated) and 969 (389 up regulated and 580 down regulated) at 6, 12 and 24 hpi,

respectively. For *Pba* SCRI1043ΔHAI2, the total number of DEGs were 242 (93 up regulated and 149 down regulated), 446 (194 up regulated and 252 down regulated) and 960 (425 up regulated and 535 down regulated) at 6, 12, and 24 hpi, respectively. Like treatment with bacteria, treatment with exogenous CFA resulted in the greatest number of DEGs at 24 hpi (a total of 211, 461 and 976 at 6, 12 and 24 hpi, respectively). The total number of DEGs observed in response to mock-inoculation control was consistently lower (a total of 88, 91, and 81 genes at 6, 12 and 24 hpi, respectively) than in tubers inoculated with bacteria. The main scope of this study was to identify the differential expression relating to bacterial invasion, hence gene isoforms and novel transcripts were not studied.

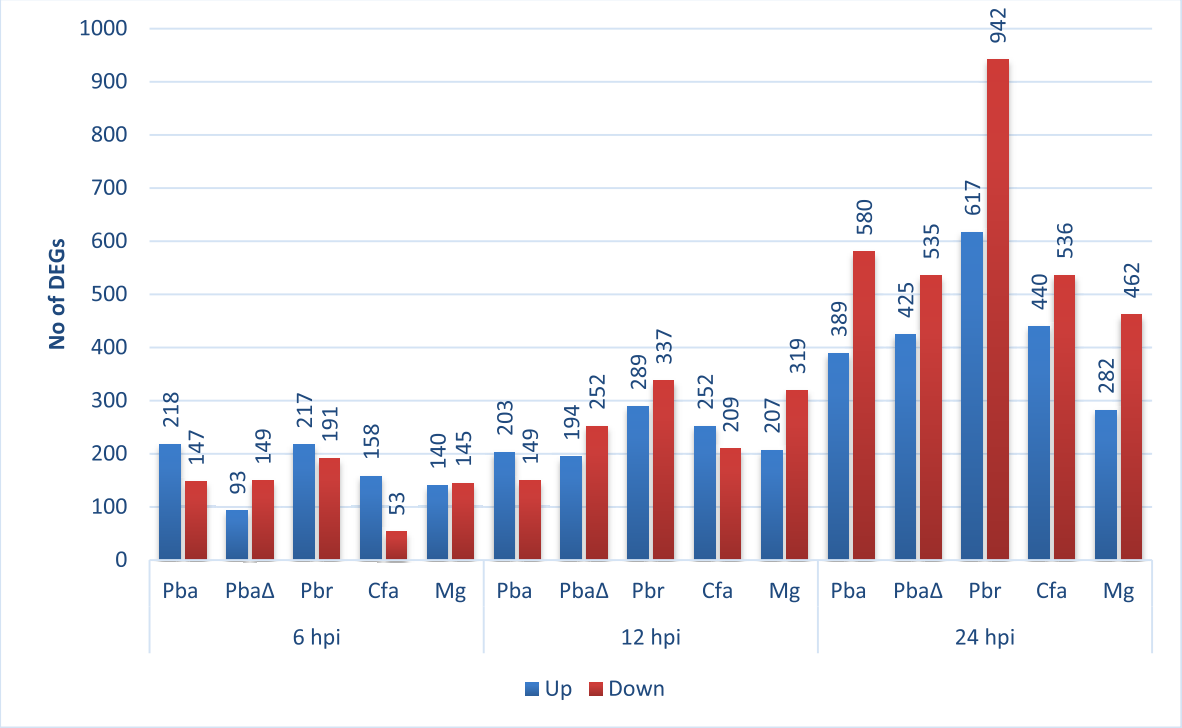


Figure 3.4: A bar graph showing the number of differentially expressed genes (DEGs) (based on log2 fold change and p_{adj} cut-off) in potato tubers ('Summer Delight') at 6, 12 or 24 hpi with either *Pbr* ICMP19477 (Pbr), *Pba* SCRI1043 (Pba), *Pba* SCRI1043ΔHAI2 (PbaΔ), exogenous Coronafacic acid (CFA) or in mock-inoculated controls (Mg) using Cuffdiff2.
Blue and red bars represent the number of genes up regulated and down regulated, respectively when compared to the non-inoculated control.

3.3.2.3.2 Differential gene expression analysis using DESeq2

Transcriptome profiling using DESeq2 revealed a total of 4347 DEGs in tubers exposed to bacteria, CFA or mock-inoculation when compared to the non-inoculated control (at one or more sampling times). Consistent with Cuffdiff2, the dynamic range of DEGs varied substantially with the time of sampling, DESeq2 revealing that differential gene expression was greatest in potato tubers at 24 hpi with bacteria (Figure 3.5). The total number of DEGs was consistently lower in mock-inoculated controls (a total of 113, 81, 199 genes at 6, 12 and 24hpi, respectively) than in tubers inoculated with bacteria.

For tubers inoculated with bacteria, the number of DEGs was greatest in tubers exposed to *Pbr* ICMP19477 regardless of sampling time (Figure 3.5). No significant differences were observed in the total numbers of DEGs in potato tubers treated with *Pba* SCRI1043 or *Pba* SCRI1043ΔHAI2. The total numbers of DEGs in response to *Pba* SCRI1043 were 107 (76 up regulated and 31 down regulated), 122 (64 up regulated and 58 down regulated) and 442 (151 up regulated and 291 down regulated) at 6, 12 and 24 hpi, respectively (Figure 3.5). For *Pba* SCRI1043ΔHAI2, the total number of DEGs were 70 (36 up regulated and 34 down regulated), 179 (82 up regulated and 97 down regulated) and 459 (199 up regulated and 260 down regulated) at 6, 12, and 24 hpi, respectively. Differential expression in potato tubers in response to exogenous CFA also varied significantly between the sampling times. Consistent with the differential expression observed in response to bacteria, a marked increase in DEGs at 24 hpi was observed. The total number of DEGs in response to CFA was 61 (47 up regulated and 14 down regulated), 141 (52 up regulated and 91 down regulated) and 496 (194 up regulated and 302 down regulated) at 6, 12 and 24 hpi, respectively.

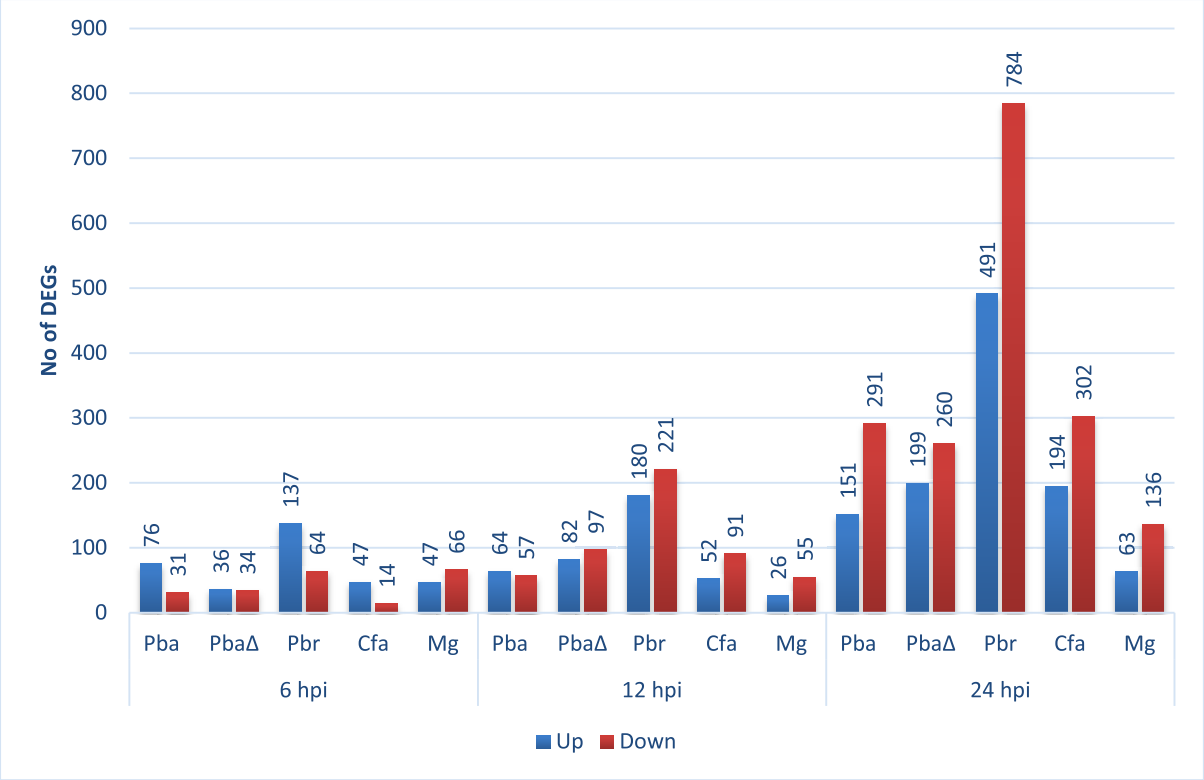


Figure 3.5: A bar graph showing the number of DEGs (based on log2 fold change and p_{adj} cut-off) in potato tubers ('Summer Delight') at 6, 12 or 24 hpi with either *Pbr* ICMP19477 (Pbr), *Pba* SCRI1043 (Pba), *Pba* SCRI1043ΔHAI2 (PbaΔ), exogenous Coronafacic acid (CFA) or in mock-inoculated controls (Mg) using DESeq2.
Blue and red bars represent the number of genes up regulated and down regulated, respectively when compared to the non-inoculated control.

3.3.2.3.3 Comparison of differentially expressed genes identified using Cuffdiff2 and DESeq2.

Comparison of the DEGs identified by Cuffdiff2 and DESeq2 revealed that across each treatment and sampling time, on average 83% of DEGs identified by DESeq2 were also identified as DE in Cuffdiff2 analysis (Figure 3.6). Cuffdiff2 always identified greater numbers of DEGs than DESeq2, however (Figure 3.6). For example, the total number of DEGs identified by Cuffdiff2 in response to *Pba* SCRI1043 at 6 hpi was 365 genes, significantly higher than the number of DEGs identified by DESeq2 (107 genes) (Figure 3.6).

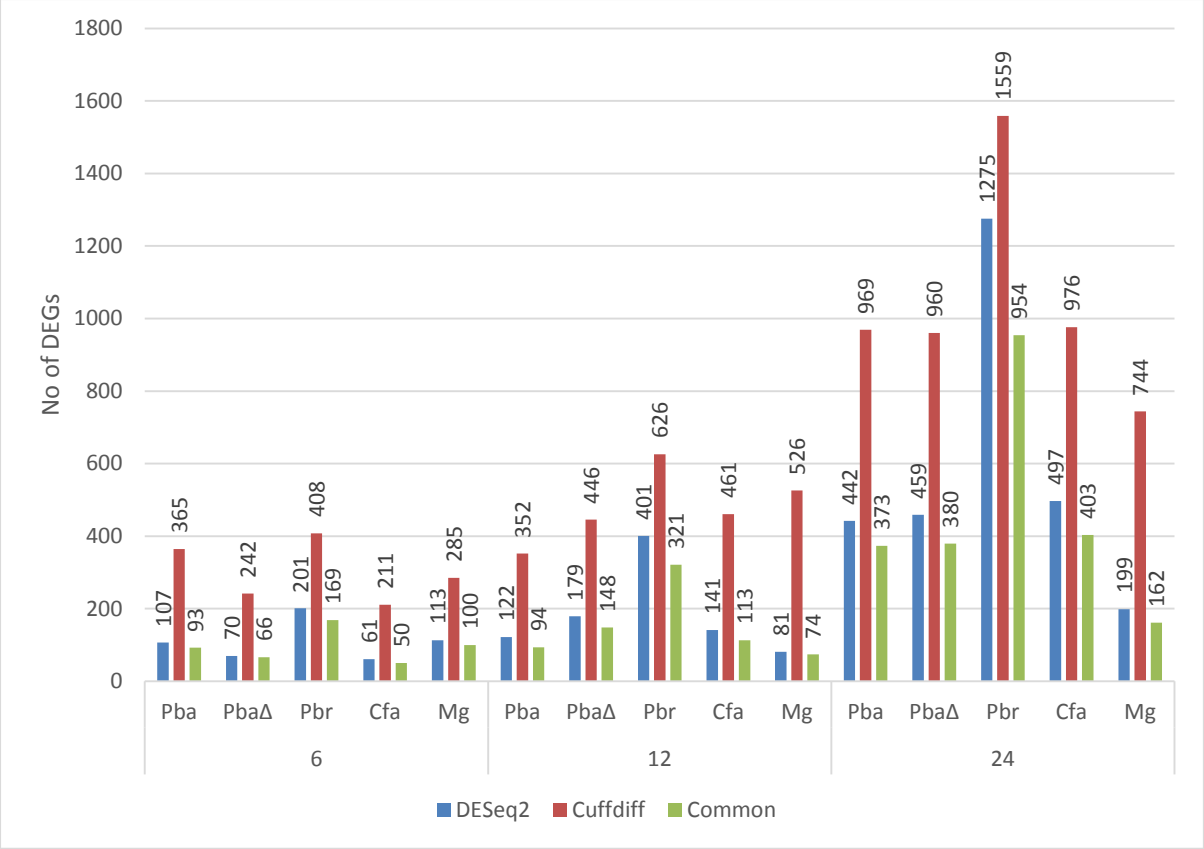


Figure 3.6: A bar graph showing the number of differential expressed genes (DEGs) (based on log2 fold change and p_{adj} cut-off) in potato tubers ('Summer Delight') at 6, 12 or 24 hpi with either *Pbr* ICMP19477 (Pbr), *Pba* SCRI1043 (Pba), *Pba* SCRI1043ΔHAI2 (PbaΔ), exogenous Coronafacic acid (CFA) or in mock-inoculated controls (Mg) using either DESeq2 (blue) or Cuffdiff2 (red). The green bars represent the number of DEGs identified by both software packages

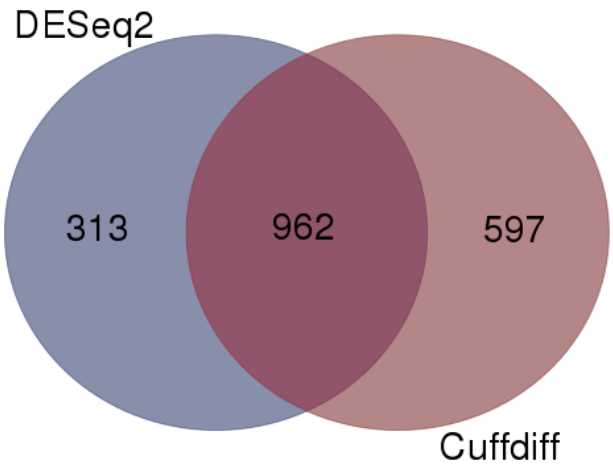


Figure 3.7: A Venn diagram showing the number of DEGs in potato tubers at 24 hpi with *Pbr* ICMP19477, as identified by DESeq2 (blue), Cuffdiff2 (pink) or both software packages.

Further to the quantitative analysis, the log2 fold change detected by DESeq2 and Cuffdiff2 analysis were compared. The DEGs obtained using DESeq2 and Cuffdiff2 analysis in response to *Pbr* ICMP19477 at 24 hpi was used as a test case. A total of 1275 and 1559 genes were identified to be DE using DESeq2 and Cuffdiff2 analysis, respectively (Figure 3.7). Of these genes identified by DESeq2 or Cuffdiff2, 962 genes were identified to be DE using both methods of analysis (Figure 3.7). Comparison of the log2 fold change of the 10 most highly down regulated and up regulated DEGs common to both analyses showed that the log2 fold change calculated by Cuffdiff2 analysis was consistently higher compared than that calculated by the DESeq2 analysis (Table 3.3). This may be why the number of DEGs identified by Cuffdiff2 was consistently higher than the number identified using DESeq2 (i.e. more genes went over the log 2 fold change threshold).

In addition to the fold changes, the count data for reads mapping to the potato genome in tubers inoculated with *Pbr* ICMP19477 and the non-inoculated control samples calculated by HTSeq-count (DESeq2) and Cufflinks (Cuffdiff2) were compared (Appendix 3.2). These comparisons showed that the FPKM values for many of the genes identified as highly DE only by Cuffdiff2 were variable across the replicates (Appendix A.2 and Appendix A.3). For example, FPKM value for the gene annotated as AP2/ERF domain-containing transcription factor (PGSC0003DMG400002899) was observed to be variable across the three replicates (0.4, 0.5 and 22). Consistently the FPKM observed in one replicate was significantly higher than the others, this resulted in a higher log2 fold change. This variability between the replicates resulted in removal of such genes from the analyses performed by DESeq2, but Cuffdiff2 averaged the FPKM values for all replicates including outliers. Thus, this inability to account for such anomalies (or outliers) might have led to errors in the log2 fold changes generated by Cuffdiff2 and to a higher rate of false positives.

Table 3.3: Log2 fold changes for the 10 most DEGs in potato tubers 24 hpi with *Pbr* ICMP19477, as identified by Cuffdiff2 or DESeq2.

Gene ID	Gene Name	Log 2fold change	
		Cuffdiff2	DESeq2
Up regulated			
PGSC0003DMG400010283	Class I chitinase	9.0	6.4
PGSC0003DMG400010310	Cytochrome P450 hydroxylase	6.1	5.0
PGSC0003DMG400038451	Pyruvate decarboxylase	5.1	4.5
PGSC0003DMG400024967	Peroxidase	5.9	4.5
PGSC0003DMG400004037	ATP binding protein	5.5	4.4
PGSC0003DMG400024771	Conserved gene of unknown function	5.3	4.4
PGSC0003DMG400022217	Inducible plastid-lipid associated protein	2.6	4.3
PGSC0003DMG400026346	F-box family protein	5.1	4.3
PGSC0003DMG400013402	Transcription factor TSRF1	5.1	4.2
PGSC0003DMG400019824	JA-induced WRKY protein	4.7	4.1
Down regulated			
PGSC0003DMG400027194	Serine/threonine-protein kinase Nek8	-6.1	-5.8
PGSC0003DMG400013413	Chlorophyll a/b-binding protein PS II-Type I	-6.0	-6.0
PGSC0003DMG400021676	Indole-3-acetic acid-amido synthetase GH3.5	-6.0	-4.5
PGSC0003DMG400042498	Chlorophyll a/b binding protein	-5.9	-5.0
PGSC0003DMG400013411	Chlorophyll a-b binding protein 3C, chloroplastic	-5.8	-5.3
PGSC0003DMG400013460	Chlorophyll a-b binding protein 3C, chloroplastic	-5.7	-5.4
PGSC0003DMG401000594	Flavonol synthase/flavanone 3-hydroxylase	-5.7	-4.3
PGSC0003DMG400020906	Plasma membrane intrinsic protein	-5.5	-4.3
PGSC0003DMG400008298	Chlorophyll a/b binding protein	-5.5	-4.7
PGSC0003DMG400003522	Galactinol synthase	-5.5	-4.7

3.4 Discussion

In this chapter, the dynamics of the interaction between a susceptible cultivar ('Summer Delight') and two of the primary SRE responsible for disease on potato were established as a prelude to transcriptional studies to understand their interactions at the molecular level. Initially, pathogenicity assays were conducted on potato tubers to identify their susceptibility to soft rotting caused by these pathogens. In these assays, the tuber weight loss caused by *Pbr* ICMP19477 was significantly greater than that resulting from inoculation with *Pba* SCRI1043, showing that *Pbr* ICMP19477 was more aggressive than its counterpart. The data obtained from this study corresponds with the results of a previous screen of New Zealand cultivars and their susceptibility to soft rot infection by *Pba* SCRI1043 and *Pbr* ICMP19477, in which *Pbr* ICMP19477 was identified as aggressive across all cultivars (Ramakrishnan, 2012).

Furthermore, *Pbr* ICMP19477 was previously identified as more aggressive on potato than strains belonging to other species and subspecies of pectobacteria in New Zealand (Pitman et al., 2008). *Pbr* is considered an aggressive tuber pathogen in South Africa causing blackleg disease and soft rot of tubers (van der Merwe et al., 2010). Assays performed on Brazilian isolates have also shown *Pbr* to be more aggressive than *Pba* under lab conditions (Duarte et al., 2004). More recently, Dutch seed production confirmed the increased incidence of *Pbr* in fields with 67% of diseased plants infected with *Pbr*, significantly higher than the infection caused by *Dickeya* spp. (21%) and *Pba* and *Pw* (6%) (*New bacteria main cause of blackleg in Dutch seed potatoes*, 2016). Taken together these data indicate that the ubiquitous nature of *Pbr* and its increased incidence in fields might be associated with its greater aggressiveness.

Despite greater maceration of potato tubers in the soft rot assays, *Pbr* ICMP19477 showed little difference in population size compared to *Pba* SCRI1043 at any given sampling time. Genome sequencing of *Pba* SCRI1043 and *Pbr* ICMP19477 has led to the identification of the variability in the number of PCWDEs produced by these pathogens (Bell et al., 2004; Panda, 2014). At least 64 putative PCWDEs have been identified in the *Pbr* ICMP19477 which is significantly higher than the 20 PCWDEs identified in *Pba* SCRI1043 (Bell et al., 2004; Panda, 2014). PCWDEs are the key virulence factors responsible for the necrotrophic life style of this pathogen (Panda, 2014). Furthermore, the release of PCWDEs in *Pectobacterium* spp. is regulated by a variety of control systems including population density dependent quorum sensing (QS) (Jones et al., 1993; Liu et al., 2008). The QS in pectobacteria is suggested to prevent the premature release of the PCWDEs and thus to prevent the activation of the plant defence response (Mae et al., 2001; Salmond et al., 1995). Studies have also proposed QS as an essential component of the plant virulence system controlling various virulence determinants, enabling the pathogen to navigate the successful transition from the asymptomatic biotrophic stage to the symptomatic necrotrophic stage of infection (Liu et al., 2008). Thus, increased maceration on potato tubers independent of greater population density is probably due to the additional PCWDEs produced by *Pbr* ICMP19477. Taken together, the results from the pathogenicity assay and the growth dynamics suggest a greater DAMP-mediated defence response during the potato-*Pbr* ICMP19477 interaction was likely.

To study the impact of CFA on the interactions between potato and *Pba* SCRI1043 or *Pbr* ICMP19477, we also compared the virulence of *Pba* SCRI1043ΔHAI2 to that of *Pba* SCRI1043. Despite the removal of HAI2 from the genome of this bacterium and consequently the loss of the CFA biosynthetic cluster housed on the island (Richter et al., 2014; Vercoe et al., 2013), the mutant showed no difference in maceration of tuber tissue when compared to the wild-type. Nor did it show any significant differences in growth upon inoculation into the tubers. These observations were in contrast to previous reports, in which deletion of CFA resulted in reduced virulence of *Pba* SCRI1043 (Bell et al., 2004; Panda et al., 2016). Virulence was assessed in stems of the host in these assays, and was performed on a different cultivar. Perhaps these experimental differences resulted in the differences observed in this study.

To support the study on the impact of CFA using SCRI1043ΔHAI2, a source of purified CFA was obtained for exogenous application to tubers. Unfortunately, initial attempts to purify CFA from cultures of *Pba* SCRI1043 were unsuccessful, so CFA was obtained from stocks previously extracted from cultures of *P. syringae* instead (kindly provided by Robin Mitchel and David Greenwood; Plant and Food Research, New Zealand). The high similarity (~75%) between the amino acid sequences of the CFA molecules predicted to be produced by *Pba* SCRI1043 and *P. syringae* (Panda et al., 2016), suggested that the transcriptional responses observed in potato exposed to this source of CFA might be similar to those obtained from CFA originating from cultures of SRE.

In the absence of evidence showing the concentration of CFA produced by SRE *in planta* (which remains unknown for *P. syringae* also), to identify a possible dose to use in the transcriptional study, CFA was applied to potato tubers at multiple concentrations to establish whether any symptoms arose. In these experiments, no major symptoms were detected, consistent with observations in previous studies (e.g. no chlorosis;) (Uppalapati et al., 2005). A small necrotrophic-like lesion was detected at the point of inoculation, however, which could have occurred in response to the putative toxin. Previously, it has been shown that, although CFA is a component of COR, it is COR that induces symptoms in the host plant including inhibition of root elongation and chlorosis (Brooks et al., 2005; Elizabeth & Bender, 2007; Feys et al., 1994; Kloek et al., 2001; Palmer & Bender, 1995; Zhao et al., 2003). In addition, purified COR induces hypertrophy in potato tissues (Sakai et al., 1979), a visual effect

that has been used as a bioassay for COR activity (Völksch et al., 1989). However, studies to test for the bioactivity of CFA in the culture supernatant of *S. scabies* confirmed that this molecule elicits no hypertrophy in potato (Fyans et al., 2015).

Regardless of the lack of a disease symptoms, given that CFA had been shown to contribute to changes in molecular signalling in tomato (Uppalapati et al., 2005), it was hypothesised that CFA would have a similar effect on transcription in potato. Indeed, it was thought that the lack of any major changes in disease symptoms might actually enable the true molecular targets to be identified more readily, especially given that large scale transcriptional changes associated with differences in the disease state of the tubers would not be expressed, and would therefore not mask more subtle hormonal modifications predicted by the structure of CFA. Furthermore, given no significant difference in nectrotic lesions were observed relative to CFA concentration, a concentration of 200 nm CFA was used for the RNA-seq experiment. This concentration was considerably higher than the dose previously used in tomato leaves (0.2 nM) (Uppalapati et al., 2005) because the tissues were sampled distal to the sight of inoculation in this study whereas the inoculation site was sampled directly in the tomato study (Uppalapati et al., 2005).

As a result of the preliminary experiments, a potato transcriptome study was conducted using the various bacteria to inoculate potato tubers or by applying exogenous CFA. Total RNA was extracted from potato tubers at 6, 12 and 24 hpi. These sampling times were identified from growth assays to represent an early period during infection of the host, when pathogen populations were rapidly expanding from the densities similar to those used for inoculation to maximal densities of 10^8 cells. Such early sampling times have previously proven successful in studying the transcriptional responses of potato and other hosts to a myriad bacterial and fungal pathogen (Gao et al., 2013; Massa et al., 2011; Petek et al., 2014; Zuluaga et al., 2015).

RNA-seq was subsequently used to obtain read data from potato tuber tissue surrounding the site of infection with the various strains of *Pectobacterium* and with exogenous CFA. The reliability of RNA-seq results, however, depends on the length of the reads and depth of the sequencing (Sims et al., 2014). The length of the reads and the quality of the mapping determines the splice variant detection (Chhangawala et al., 2015; Sims et al., 2014). As a

result, the longer the reads, the more likely the origin of the transcript is to be identified. Sequence depth (coverage) also defines the dynamic range of the transcript profile that can be extracted from a sample (Sims et al., 2014). Consistent with the theoretical performance parameters promised by Illumina HiSeq 2000, the number of reads and the read lengths obtained in this study met the criteria required for downstream analysis. In fact, the average read length of 86 bp obtained post quality control analysis promised highly effective mapping to the Potato reference genome (Sharma et al., 2013).

Despite good read coverage and length, the percentage of reads mapping to the potato genome (70%) was relative low compared to the number reported in other potato transcriptomics studies (80-85%) (Gao et al., 2013; Yogendra & Kushalappa, 2016). The reason for this unexpectedly low result remains unknown, but it was speculated that the age of potato tubers and the tissue source might explain the differences in the mapping efficiency. For example, studies using fresh potato tubers have on average mapped 90% of reads whereas transcriptome data of tubers in cold storage have routinely generated ~70% coverage (Susan Thomson, Personal communication). Additional scrutiny of the RNA-seq datasets using PCA analysis revealed 65% of the variance, where PC1 was indicative of sampling time (51%) and PC2 was indicative of treatment (14%). This meant a substantial proportion of the variance in this experiment was probably due to sample variation. This variability was visibly greater in the later sampling times, probably due to the variation in the rate of disease progression between the replicates. For example, at the 12 hpi sampling time when the plant and the pathogen are still competing to establish resistance and susceptibility, respectively. With the variance in the experiment in mind, although it is crucial to access the global quality of the RNA-seq data set, no clear thresholds exist to define whether biological variability between samples is acceptable, especially as this depends on the heterogeneity of the organism. Potato is often considered to be highly heterogeneous (Bamberg et al., 2015; Potato Genome Sequencing Consortium, 2011), suggesting that the experiments using this host are likely to have high levels of variance.

RNA-seq analysis generated a large set of data of the transcriptional changes in distal tuber tissue after inoculation with *Pbr* ICMP19477, *Pba* SCRI1043 and *Pba* SCRI1043ΔHAI2 or after injection of exogenous CFA. A quantitative analysis of these data (presented in Figure 3.4 and 3.5) confirmed that the statistically significant DEGs in response to all three bacterial strains peaked at 24 hpi. Furthermore, a significant number of genes were down regulated in

response to the bacteria at this sampling time. The 24 h sampling time corresponds to a time when high cell densities have developed in the plant, suggesting that the plant is undergoing major transcriptional reprograming due to being overridden by the bacteria. In addition, *Pbr* ICMP19477-mediated differential expression was greater across all time points when compared to the other treatments. Greater differential expression in response to this pathogen may have been due to the highly aggressive nature of the pathogen and the greater extent of cell wall degradation by this pathogen. In turn, this would release greater quantities of cell wall components that are known to elicit a DAMP-mediated response in plant hosts (Boller & Felix, 2009; Galletti et al., 2009).

Finally, the major aim of this chapter was to compare the two software packages (DESeq2 and Cuffdiff2) that were available for analysing the read data generated by this RNA-seq experiment. In a preliminary study conducted during my MSc study, Cuffdiff was used for differential expression analysis (Ramakrishnan, 2012). Other RNA-seq comparative studies, however, have shown differences in the prediction of DEGs by different software packages, using real and simulated datasets for direct assessments of the sensitivity and specificity of the differential expression detection as well as the FDR control (Burden et al., 2014; Guo et al., 2013; Kvam et al., 2012; Rapaport et al., 2013; Schurch et al., 2015; Seyednasrollah et al., 2015; Soneson & Delorenzi, 2013; Zhang et al., 2014). When compared to other RNA-seq analysis packages (including DESeq), Cuffdiff and Cuffdiff2 have been identified to perform poorly, possibly due to the transformation of the alignment results to FPKM values, rather than raw read counts (Conesa et al., 2016; Seyednasrollah et al., 2015; Zhang et al., 2014). Several studies have also indicated that FPKM may not be an appropriate way to normalise RNA-seq data (Giorgi et al., 2013; Li et al., 2012). Furthermore, a recent study, also suggested that the transcript length used by Cuffdiff2 may not be an appropriate approach and could potentially introduce conservative bias in the analysis (Di et al., 2011). In this study, although most of the DEGs identified by DESeq2 and Cuffdiff2 overlapped (< 80% of DEGs identified by DESeq2 were typically detected using Cuffdiff2), Cuffdiff2 always detected a greater number of DEGs. These results were consistent with previous studies, in which a comparison between the DEGs identified by Cuffdiff2 and DESeq2 showed Cuffdiff2 introduced the most false positives (Rajkumar et al., 2015). Interestingly, among the DEGs, many genes with high fold change were observed using Cuffdiff2 software but not by DESeq2. Further, FPKM analysis

confirmed that either these DEGs had small FPKM values or were highly variable between the biological replicates. In contrast, the inability to detect these DEGs in DESeq2 analysis was probably partly due to the conservative approach used by DESeq2 for DE detection. In particular, DESeq2 detects outliers within the biological replicates and subsequently removes the affected genes from downstream analysis. This significantly reduces the false positives produced by the analysis and the higher fold changes between treatments (Love et al., 2014). Thus, the results observed in this comparative analysis were in agreement with the previous studies, in which DESeq/DESeq2 was identified as more conservative than other count base tools (Kvam et al., 2012; Seyednasrollah et al., 2015; Soneson & Delorenzi, 2013; Zhang et al., 2014).

Overall, the comparison of the two differential expression analysis packages suggested that there can be large differences in their identification of DEGs, and that even different versions of the same package can alter the outcomes of the analysis (data not shown). As a result, documenting the settings, scripts and the version numbers of the programs appears essential to ensure reproducibility of the experiments. In addition, repetition of the differential expression analysis with different software packages would provide a better understanding of the statistical power required for differential expression detection. Nevertheless, in this study, DESeq2 was used for further functional and pathway analysis, as the software was considered to use a more stringent approach for differential expression detection (Kvam et al., 2012; Seyednasrollah et al., 2015; Soneson & Delorenzi, 2013; Zhou et al., 2011). Data was, however, scrutinized manually to ensure important non-statistical or highly variable transcriptional changes were incorporated into the interpretations of the data. This approach was predicted to reduce the high levels of background noise and variability associated with sequencing and analysis packages respectively, which might mask the subtle impacts of CFA on the infection process. Given that transcriptome studies often report primarily on the most DEGs, our comparison confirmed the variability between DEGs identified between different packages and emphasised the importance of pathway analysis (MapMan or KEGG analysis) in order to fully understand the global view of the transcriptional changes occurring under various biotic stresses.

3.5 Conclusion

The capacity of *Pbr* ICMP19477 and *Pba* SCRI1043 to cause soft rot in 'Summer Delight' was compared to the maceration caused by a mutant of *Pba* SCRI1043 unable to produce the virulence factor CFA. Colonisation of the host tissue was shown to be similar for all strains, though *Pbr* ICMP19477 produced greater tissue maceration. This information identified suitable times for total RNA extraction to study the host response to infection, but also provided background knowledge to support the future interpretation of the transcriptome data. Furthermore, the comparison of the software tools for differential gene expression analysis confirmed DESeq2 as a stringent method, suitable for studying differential expression in potato without generating the high levels of background noise associated with Cuffdiff2, which might mask the subtle impacts of CFA on the infection process.

Chapter 4 Differential expression in potato tubers in response to *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* subsp. *brasiliensis*

4.1 Summary

Having identified DESeq2 as an appropriate software package for generating differential expression data from Illumina HiSeq RNA-seq in Chapter 3, DESeq2 was used to ascertain the global systemic transcriptional profiles of potato tubers ('Summer Delight') upon infection with *Pbr* ICMP19477 or *Pba* SCRI1043. Consistent with the production of a plethora of PCWDEs by both pathogens, infection with either strain induced a characteristic DAMP-mediated PTI defence response, with early defence response genes involved in the oxidative burst, ET biosynthesis and signalling, JA signalling and GA biosynthesis showing significant differential expression. Secondary metabolism was also induced, genes involved in the synthesis of terpenoids showing significant up regulation in response to both SRE. Of particular note, infection with *Pectobacterium* led to the up regulation of genes related to phytoalexin biosynthesis, including divinyl ether synthase *(des)*, which is involved in the synthesis of the antimicrobials colneleic and colnelenic acid. The PTI response was of greater magnitude upon infection with *Pbr* ICMP19477 too, with both the number of DEGs in each pathway and the level of differential expression (as defined by the log2 fold change) greater in response to this aggressive strain. In addition, two snakin genes (*Sn-1* and *Sn-2*) were amongst those with greatest up regulation in response to this pathogen. *Sn-1* and *Sn-2* were previously engineered for expression under an inducible promoter, which resulted in quantitative resistance to SRE in potato. Interestingly, given that a single effector (DspE/A) has been identified in *Pba* SCRI1043 and *Pbr* ICMP19477, NBS-LRR genes related to effector perception appeared to be significantly induced only in response to the latter strain. In particular, a subgroup of NBS-LRR genes annotated as encoding Avr9/Cf-9-like proteins were up regulated. *Avr9/Cf-9* was the first identified fungal avirulence gene, which elicits race-specific resistance to *Cladosporium fulvum*. Thus, differential expression analysis identified

key defence-related pathways potentially associated with both PTI and ETI that were triggered by one or both of these strains. These findings led to preliminary screening of the secondary metabolites produced in response to these SRE using mass spectrometry.

4.2 Background

Phytopathogenic bacteria use various strategies to colonize plants and derive nutrition from their hosts (Davidsson et al., 2013), but based on their mode of association with host cells they have been classified into three broad classes. Biotrophic phytopathogens, use stealth-like strategies to manoeuvre host defence systems to maintain host viability and thrive on nutrients from living host cells (Glazebrook, 2005). They largely depend on their ability to avoid plant detection and to suppress host defence responses (Collmer et al., 2009; Göhre & Robatzek, 2008; Kay & Bonas, 2009). In contrast, necrotrophic phytopathogens use brute force strategies, actively deploying PCWDEs, toxins and necrosis-inducing proteins to elicit cell necrosis and cause leakage of nutrients that lead to the death of host tissues (Davidsson et al., 2013; Glazebrook, 2005). Though most pathogens have historically been grouped into one of these two categories, some phytopathogenic bacteria possess both biotrophic and necrotrophic characteristics. Indeed, phytopathogenic bacteria are increasingly considered to be hemibiotrophs. Hemibiotrophic pathogens exhibit distinct phases in their life cycles; an early biotrophic phase where they obtain nutrients from the tissue and a late necrotrophic phase where they secrete virulence proteins leading to cell/tissue death (Glazebrook, 2005; Lee & Rose, 2010). The duration of the biotrophic phase versus the necrotrophic phase varies among pathogens. Biotrophic and hemibiotrophic pathogens often display high degrees of host specificity (Lindeberg et al., 2012; Niks & Marcel, 2009). In contrast, most necrotrophic pathogens have broad host ranges, though some are also host-specific (Mengiste, 2012). Host-specific necrotrophs like *Cochliobolus carbonum* produce specific toxins essential for their pathogenicity in the host cell (Walton, 1996).

Pectobacteria were typically considered broad host necrotrophs that used brute force strategies to overcome their hosts, employing PCWDEs and other necrosis-inducing proteins

to macerate host tissues and feed on the nutrients released (Czajkowski et al., 2012; Perombelon, 2002). The ability of SRE to live in plant tissues without causing symptoms, however, suggested that they also utilised an asymptomatic biotrophic phase during infection (Toth & Birch, 2005). Thus, pectobacteria became more widely thought of as hemibiotrophic pathogens (Davidsson et al., 2013; Liu et al., 2008; Toth & Birch, 2005). As hemibiotrophic pathogens, after latent colonisation of the plant, changes to environmental conditions favouring bacterial growth to high cell densities (typically including high moisture and low oxygen) appeared to result in the SRE initiating PCWDE production and the necrotrophic phase of their lifecycle (Perombelon, 2002; Perombelon & Kelman, 1980). Indeed, comparative genomics of *Pba* and *Pbr* showed that these SRE shared a common cluster of PCWDEs, which formed part of the core genome (Glasner et al., 2008; Panda, 2014). In addition to the similarities in the PCWDEs, certain species-specific differences existed. For example, in addition to 64 predicted PCWDEs, *Pbr* ICMP19477 encodes a novel M20 family peptidase, PepV and an additional rhamnogalactoronate lyase (Panda, 2014).

The switch from a latent phase to a necrotrophic phase in SRE is believed to be dependent on virulence factors other than the PCWDEs, but an understanding of how these virulence factors operate remains elusive.

The T3SS, however, is a protein translocator machinery involved in delivering effectors into the plant cell that are required for the virulence of many biotrophic and hemibiotrophic pathogens. Interestingly, the composition and organisation of the T3SS proteins is mostly conserved across SREs (Glasner et al., 2008; Panda, 2014). Furthermore, the inactivation of the T3SS machinery in some strains results in delayed infection, suggestive of T3SS-mediated host defence suppression (Holeva et al., 2004a; Yang et al., 2002). Unlike *P. syringae*, however, *Pectobacterium* spp. have relatively few T3-secreted effector proteins (Glasner et al., 2008; Kim et al., 2011; Kim et al., 2009). In fact, DspE is the only effector protein known to be secreted by the T3SS in pectobacteria. Further characterisation of DspE showed that the T3 effector induces cell death and tissue maceration, characteristic of necrotrophic infection rather than an effector induced HR (Hogan et al., 2013; Kim et al., 2011; Kim et al., 2009). Furthermore, DspE is smaller than homologues in the closely related phytopathogen *Erwinia amylovora*, and unlike its alleles, is unable to inhibit callose deposition, an effector-mediated

susceptibility observed in the host (Kim et al., 2011). Gene expression profiles observed in tobacco in response to *Pcc* also show a defence response consistent with that induced in response to a *P. syringae* T3SS mutant. Furthermore, infection by *Pcc* fails to suppress the PTI-mediated defence response, including the basal defence response (Kim et al., 2011). Consequently, a comparison of virulence between wild-type *Pcc* strains and naturally T3SS-deficient *Pcc* strains failed to reveal any significant differences in virulence (Kim & Salzberg, 2011; Kim et al., 2009). Consistent with these findings, recent gene expression profiling suggested that *Pcc* uses the T3SS to induce plant cell death in order to promote tissue maceration rather than to supress the PTI-mediated defence response (Hogan et al., 2013; Kim et al., 2011).

Invasion by pathogenic bacteria activates either PTI or ETI in host plants depending on the mode of infection employed (for more detail on PTI or ETI see Sections 1.8.1 and 1.8.2, respectively). PTI is initiated by recognition of PAMPs (see section 1.8.1.1) or DAMPs (section 1.8.1.2), plant cell wall fragments that are released upon cell wall degradation. In contrast, the HR and its associated cell death, which are hallmarks of ETI-mediated defence, are usually elicited in response to biotrophic pathogens. In fact, ETI is thought to benefit necrotrophic pathogens since their success relies on host cell death (Mengiste, 2012). Given the lack of evidence for effectors encoded by pectobacteria, it is hypothesised that ETI plays a minimal role (if any) in the host's response to infection. Yet, very little is known about the defence response of the primary host to these pathogens. Instead, knowledge is limited to that acquired through studies on the non-host plant *Arabidopsis* (Norman-Setterblad et al., 2000; Po-Wen et al., 2013). In the absence of experimental data on potato, the DAMP-triggered PTI-mediated defence response is considered the most effective defence strategy to combat SRE (Davidsson et al., 2013).

In this chapter, in order to enhance our knowledge of the defences elicited in potato in response to infection with *Pectobacterium* spp., the transcriptional profiles of potato tubers ('Summer Delight') was compared upon infection with *Pba* SCRI1043 and *Pbr* ICMP19477 using DESeq2. *Pba* SCRI1043 and *Pbr* ICMP19477 were used because of their variation in the aggressiveness (as described in Section 3.3.1.1), which enabled the impact of strain variation

to be explored. It also enabled the possibility of identifying unique reactions to each strain that might be attributed to ETI (stimulated in response to cryptic effectors in either strain).

4.3 Results

Differential gene expression in potato tubers in response to *Pba* SCRI1043, *Pbr* ICMP19477 and mock-inoculation was compared across all sampling times (6, 12, and 24 hpi) using a Venn diagram (Figure 4.1). The log2 fold change and the p_{adj} value of the DEGs were then used as a threshold to generate a complete list of DEGs in these treatments (Appendix B.1).

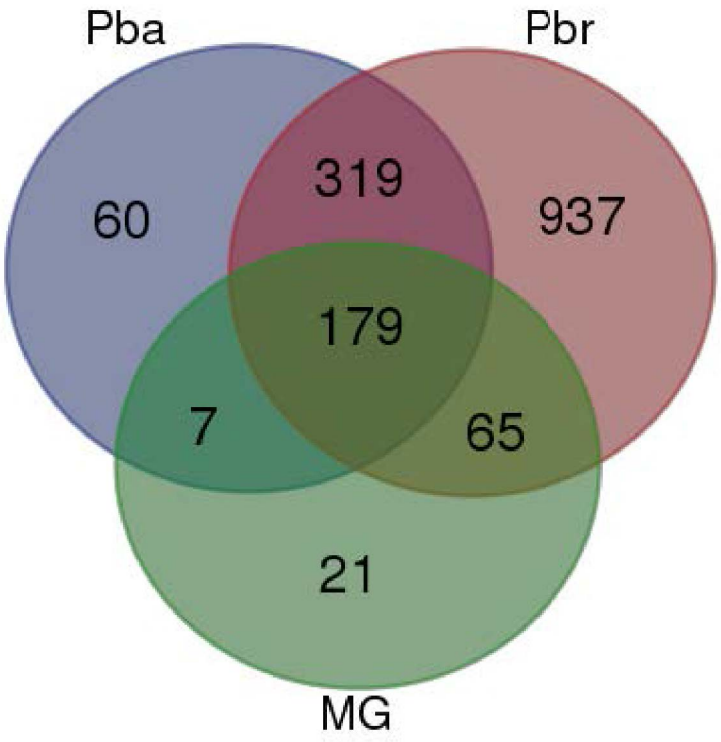


Figure 4.1: A Venn diagram showing the number of DEGs in potato tubers ('Summer Delight') in response to *Pba* SCRI1043, *Pbr* ICMP19477 and/or the mock-inoculated control (MG) across all time points.

When both a log2 fold change in differential expression and a $p_{adj} \leq 0.05$ was used as a statistical threshold, a total of 1500, 565 and 272 genes were DE in tubers in response to *Pbr* ICMP19477, *Pba* SCRI1043 and the mock-inoculation control at one or more time points, respectively (Figure 4.1). Of these DEGs, only 179 were identified as common across all treatments (Appendix B.2). In addition, 319 genes were DE in response to both *Pba* SCRI1043 and *Pbr* ICMP19477 (Appendix B.3), while a further 65 and 7 genes were DE in response to both mock-inoculation and *Pbr* ICMP19477 or mock-inoculation and *Pba* SCRI1043, respectively. A total of 937 and 60 genes were DE only in response to *Pbr* ICMP19477 or *Pba* SCRI1043, respectively, while 21 DEGs were exclusive to the mock-inoculated control at one or more time points.

4.3.1 Differential gene expression in response to wounding

Genes related to calcium influx, oxidative burst, ET biosynthesis and signalling, the abiotic stress response (such as heat shock protein) and primary metabolism (including the Calvin cycle and light reaction) were significantly DE in response to mock-inoculation and pectobacteria. In addition, differential expression of other defence-related responses such as 'transcription factors (TFs)' and 'secondary metabolite biosynthesis' were observed in response to pectobacteria, but were non-significant in response to mock-inoculation (Appendix B.2). Taken together, in this study, a significant overlap of defence-related pathways was observed in response to mock-inoculation and infection with pectobacteria. This is consistent with the previous understanding that mechanical wounding/damage caused during mock-inoculation is recognised as "damage within", resulting in DAMP-mediated PTI response. Hence, in this thesis, differential expression observed in response to mock-inoculation is explained within the context of differential expression observed in response to pectobacteria in section 4.3.3.

4.3.2 Functional annotation of the transcriptional profiling in response to *Pba* SCRI1043 and *Pbr* ICMP19477

The DEGs identified by DESeq2 were divided into gene categories to establish whether specific functional categories were over-represented in response to each treatment. Gene Ontology implemented in BLAST2GO (3.3.5) was used for this purpose. GO provides the ontology of defined terms representing gene products, dividing them into three categories: “Cellular Component”, “Biological Processes” and “Molecular Functions”. As the central objective of this thesis was to understand defence responses and signal transduction induced in potato tubers in response to *Pbr* ICMP19477 and/or *Pba* SCRI1043, the focus was on DEGs associated with “Biological Processes”. The term “Biological Processes” in plant-microbe interactions is described as a series of events or molecular functions involved in inhibition of bacterial invasion or events involved in plant defence response. Biological processes are accomplished by one or more organised assemblies with a defined beginning and end.

Approximately 35% of the DEGs in response to *Pba* SCRI1043, *Pbr* ICMP19477 and mock-inoculation were involved in biological processes. Of these, genes relating to single-organism cellular processes (31%, 40%, 36%), oxidation-reduction processes (26%, 25%, 28%), single-organism biosynthetic processes (13%, 17%, 20%), small molecule metabolic processes (14%, 15%, 8%), regulation of transcription (14%,5%, 12%), response to light (6%, 6%, 3%) and response to stress (11%, 2%, 16%) were DE in response to *Pba* SCRI1043, *Pbr* ICMP19477 and the mock-inoculation control, respectively (Figure 4.2).

Genes related to cellular protein modification (19%, 17%), carbohydrate metabolic processes (14%, 9.4%), transport (8%, 9%), transmembrane transport (2%, 2%), response to chemical (3%, 2%) and the light reaction (2% and 1%) were DE in response to both *Pba* SCRI1043 and *Pbr* ICMP19477, respectively, but not mock inoculation (Figure 4.2). Furthermore, genes related to catabolic processes (12%), multicellular organism processes (2%), secondary metabolite biosynthesis (2%), and phosphorylation (2%) were DE expressed only in response to *Pba* SCRI1043 (Figure 4.2).

The ontologies of the genes DE in response to *Pbr* ICMP19477 were diverse. This was evident from the over-representation of the other biological process categories (Figure 4.2). Interestingly, genes relating to defence response (10%), metabolic processes (including primary and cellular metabolic processes) and biosynthetic processes (including aromatic compounds (10%), nitrogen compounds (9.5%), macromolecule (9%), and heterocycle (8.5%)) were over represented only in response to *Pbr* ICMP19477 at 24 hpi (Figure 4.2). This, suggested that DE of these genes was possibly involved in the active defence response deployed by the host in response to this aggressive pathogen. Ontologies for the genes DE in response to *Pba* SCRI1043 and *Pbr* ICMP19477 at different time points are shown in Appendix B.4 and B.5, respectively.

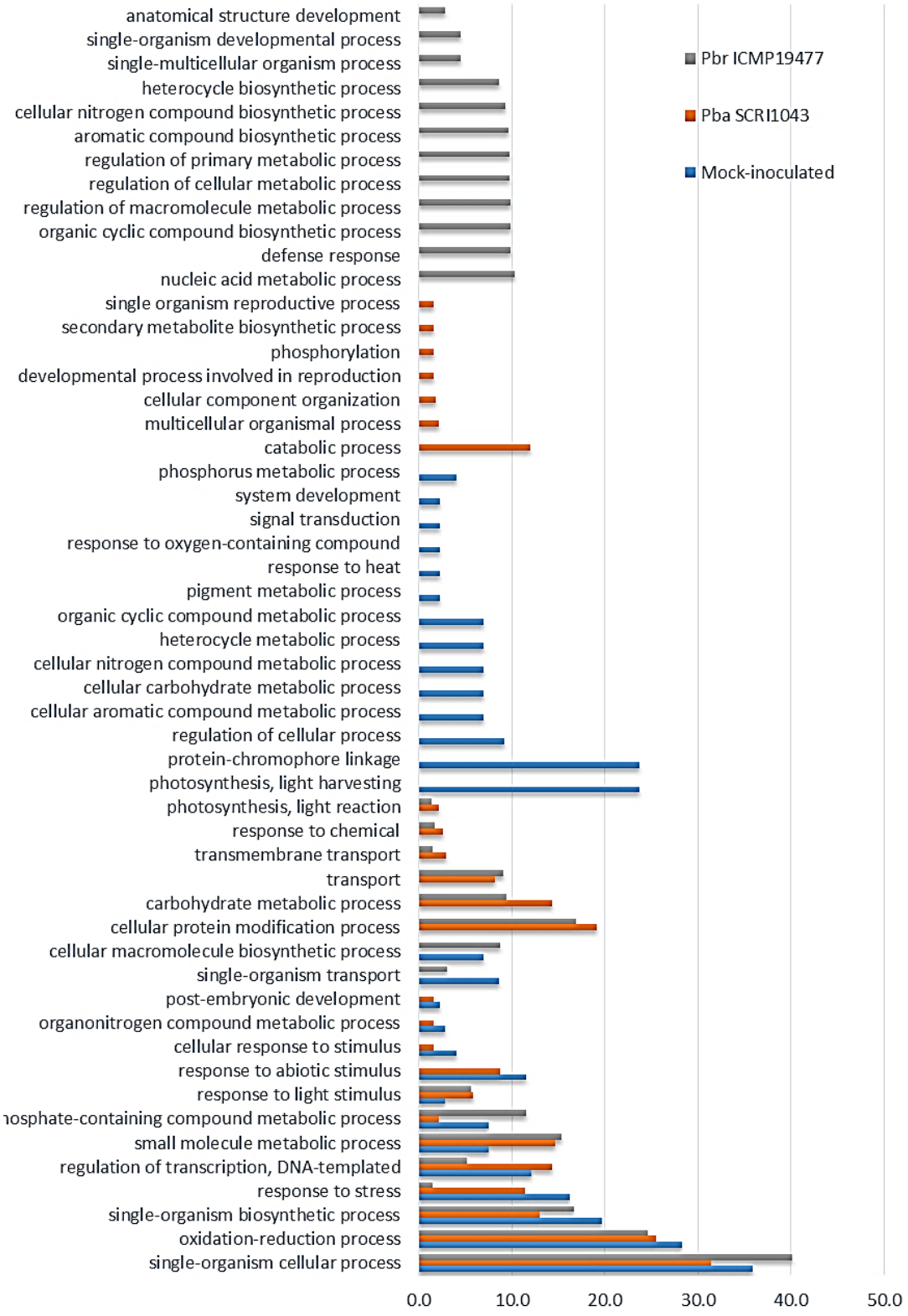


Figure 4.2: Gene ontologies of all DEGs involved in the "biological processes" in response to mock-inoculation (blue), *Pba* SCRI1043 (orange) and *Pbr* ICMP19477 (grey) across all sampling time.

BLAST, InterPro scan, and GO annotation implemented in BLAST2GO (3.3.5) were used to construct functional categories for DEGs. X axis represents the percentage of genes present in each category.

4.3.3 Differential gene expression in response to *Pba* SCRI1043 and *Pbr* ICMP19477 at one or more time points.

Initial quantitative analysis identified 321 DEGs in response to *Pbr* ICMP19477 and *Pba* SCRI1043, representing the "core" response to pectobacteria. In addition, the number of DEGs observed only in response to *Pbr* ICMP19477 was higher than the DEGs observed in response to *Pba* SCRI1043. Further fold change analysis confirmed that several genes identified in response to *Pbr* ICMP19477 were also DE expressed in response to *Pba* SCRI1043, however, the fold change observed in the latter was below the significance threshold. The non-significant differential expression of some genes in response to *Pba* SCRI1043 suggested that the core response of potato tubers to *Pbr* ICMP19477 and *Pba* SCRI1043 might be larger than the stringent DESEq2 analysis had revealed.

Genes relating to a variety of pathways were DE in response to *Pbr* ICMP19477 and *Pba* SCRI1043, although not all gene categories are discussed here. Only the impact of *Pectobacterium* spp. on 'biological processes' relating to the host defence response are described. In particular, DEGs relating to the early host response (including the NBS-LRR proteins, ion influx, oxidative bust, and cell wall modification), plant hormone-induced defence responses (JA/ET, ABA and GA related signal transduction), TFs and secondary metabolite biosynthesis are reported. The MapMan analysis of the DEGs observed in response to pectobacteria and mock-inoculation is presented in Figure 4.3.

4.3.3.1 Pathogen- and Effector-triggered immunity

Receptor kinases (LRRs) and NBS-LRR resistance proteins

Several genes annotated as LRR receptor kinases (e.g. PGSC0003DMG400013804) were up regulated in response to both *Pbr* ICMP19477 and *Pba* SCRI1043 (Table 4.1). Additional receptor kinases (e.g. PGSC0003DMG400025480, PGSC0003DMG400013201) were also induced

solely by *Pbr* ICMP19477 (Table 4.1). The two remaining LRR receptor kinases (PGSC0003DMG401004603, PGSC0003DMG400028561) were down regulated. As LRR receptor kinases play a central role in recognising PAMPs and DAMPs (Dodds & Rathjen, 2010; Jones & Dangl, 2006), those induced by the two pathogens may be involved in their detection or detection of cell wall damage during infection. Expression of the down regulated receptor kinases may have been reduced to lower the energy burden on the host.

Differential expression of genes annotated as encoding NBS-LRRs was also observed. The majority were down regulated, mostly in response to *Pbr* ICMP19477 (Table 4.1). Conspicuously, however, Avr9/Cf-9 induced kinase 1 (*ACIK1*) and a number of Avr9/Cf-9-related proteins were significantly induced in response to *Pbr* ICMP19477 (Table 4.1). NBS-LRRs are considered to be associated with ETI and *Avr9/Cf9* was famously the first fungal resistance gene identified in tomato in response to *C. fulvum* (Hammond-Kosack et al., 1994; Hammond-Kosack et al., 1996; Honee et al., 1995).

Table 4.1: A list of selected DEGs associated with pathogen perception in response to *Pbr* ICMP19477, *Pba* SCRI1043 and mock-inoculation across all sampling time.

Gene ID	Transcript Name	Mock-Inoculated			*Pbr* ICMP19477			*Pba* SCRI1043		
		6 hpi	12 hpi	24 hpi	6 hpi	12 hpi	24 hpi	6 hpi	12 hpi	24 hpi
Receptor Kinase										
PGSC0003DMG400025480	Receptor protein kinase			1.1			**2.6**		1.1	
PGSC0003DMG400013201	Receptor protein kinase						**2.3**			
PGSC0003DMG400013804	Receptor protein kinase			1.7		1.1	**2.3**			1.5
PGSC0003DMG401004603	Receptor kinase 3			-1.4			**-2.4**			-1.2
PGSC0003DMG400028561	Receptor protein kinase			-1.5		-1.8	**-3.4**			-1.8
Effector triggered resistance proteins										
PGSC0003DMG400002327	Avr9/Cf-9 induced kinase 1			1.1	1.3	1.5	**3.1**	1.3	1.2	1.8
PGSC0003DMG400001396	Avr9/Cf-9 rapidly elicited protein 231		1.1	1.2		1.1	**2.8**	1.2	1.4	1.1
PGSC0003DMG400004900	Avr9/Cf-9 rapidly elicited protein 132			1.9		1.6	**2.7**		1.3	1.5
PGSC0003DMG400016149	Avr9/Cf-9 rapidly elicited protein 75						**2.3**		1.5	
PGSC0003DMG400020918	Avr9/Cf-9 rapidly elicited protein 189			1.5			**2**			1.6
PGSC0003DMG400013736	BED finger-NBS-LRR resistance protein			-1.2			**-2.2**			-1.3
PGSC0003DMG400030239	CC-NBS-LRR protein			-1.2		-1.2	**-2.2**			
PGSC0003DMG402027371	CC-NBS-LRR protein			-1.8		-1.6	**-2.6**		-1.2	
PGSC0003DMG402030236	CC-NBS-LRR protein			-1.8		-1.3	**-2.7**		-1.3	-1.1
PGSC0003DMG403008349	CC-NBS-LRR resistance protein	-1.1	-1	-1.3		-1.2	**-2.1**			-1.9
PGSC0003DMG401011427	CC-NBS-LRR resistance protein			-1.9		-1.5	**-2.1**		-1.2	-1.4
PGSC0003DMG400010944	CC-NBS-LRR resistance protein			-1.1			**-2.2**			
PGSC0003DMG400011111	CC-NBS-LRR resistance protein			-1.2			**-2.7**			
PGSC0003DMG401008349	CC-NBS-LRR resistance protein			-2		-2.4	**-3.1**		-1.4	-1.3
PGSC0003DMG402017089	NBS-coding resistance gene			-1.1		-1	**-2**			-1
PGSC0003DMG400001994	NBS-LRR protein						**-2**			

PGSC0003DMG400031881	NBS-LRR resistance protein			-1.3		**-2**		
PGSC0003DMG400020737	NBS-LRR resistance protein			-1		**-2.3**		
PGSC0003DMG400011112	NBS-LRR resistance protein			-1.4	-1.4	**-2.5**		
PGSC0003DMG400002982	Potato resistance I2GA-SH23-1			-1.1	-1.3	**-2.2**		-1
PGSC0003DMG400019669	Potato resistance I2GA-SH23-3			-1.6		**-2.7**	-1	
PGSC0003DMG400019667	Potato resistance I2GA-SH23-3			-1.3		**-2.3**		
PGSC0003DMG400022699	Resistance gene		-1	-2.1	-1.6	**-2.3**	-1.1	-1
PGSC0003DMG403016981	Resistance gene			-1.7	-1.3	**-2.4**		-1.1
PGSC0003DMG401022784	Resistance protein PSH-RGH6	-1.4		-1.3	-1.1	**-2.1**	-1	-1.4
PGSC0003DMG401009819	Resistance protein PSH-RGH6			-1.6	-1.5	**-2.4**		
PGSC0003DMG400004874	Resistance protein PSH-RGH7			-1.2	-1.2	**-2.1**		-1.4
PGSC0003DMG400027867	Resistance protein PSH-RGH7				-1.3	**-2.4**		-1.5
PGSC0003DMG401016933	Disease resistance protein			-1.9	-1.3	**-3**	-1.1	
PGSC0003DMG402018696	Disease resistance protein I2					**-2**		-1
PGSC0003DMG400018693	Disease resistance protein I2			-1.6		**-2.2**	-1.5	
PGSC0003DMG401018576	Disease resistance protein I2C-5			-1.8	-1.4	**-2.6**	-1	-1.4
PGSC0003DMG400014047	Disease resistance protein R3a			-1.7	-1	**-2.2**		
PGSC0003DMG400009455	Disease resistance protein R3a			-1.4	-1.1	**-2.3**		
PGSC0003DMG400002980	Disease resistance protein R3a			-1.4	-1.5	**-2.4**	-1.2	-1.1
PGSC0003DMG401018696	Disease resistance protein R3a		-1	-1.4	-1.5	**-2.6**		
PGSC0003DMG400035467	Disease resistance protein RGA2		-1.2	-1.7	-1.5	**-2.6**		-1.6
PGSC0003DMG403015682	Bacterial spot disease resistance protein 4			-1.3		**-2.3**	-1	

Note: The non-inoculated control was used to normalize DEGs. When a value is not present the data did not pass the quality control. The statistically significant DEGs are presented in **BOLD.** Numerical values represent the log2 fold change, positive values indicate the induced expression of the transcript when compared to the non-inoculated control and negative values indicate the repression of the transcript when compared to the non-inoculated control.

Phosphoinositide–Phospholipase C induced plant defence signalling

Genes annotated as involved in phosphoinositide-phospholipase C (PI-PLC)-induced plant defence signalling were DE in response to both *Pbr* ICMP19477 and *Pba* SCRI1043. In particular, a gene annotated as PI-phospholipase C PLC6 (PGSC0003DMG400030360), involved in the hydrolysis of phosphatidylinositol 4, 5-biphosphate (PIP2) to generate the second messengers inositol 1,4,5-triphosphate (InsP3) and Diacylglycerol (DAG), was significantly induced at 24 hpi (Table 4.2). Inositol-1,4,5-triphosphate-5-phosphatase (PGSC0003DMG400024219) and inositol polyphosphate multikinase (*InsPK*) (PGSC0003DMG400014228) were also induced in response to both strains at 24 hpi. Though genes encoding PI-phospholipase C PLC6, inositol-1,4,5-triphosphate-5-phosphatase and InsPK were also induced in mock-inoculated potato tubers, the difference in expression from the non-inoculated control was not statistically significant (either differential expression < ±2 log2 fold change or $p_{adj} \leq 0.05$). Genes encoding Phospholipase C (PGSC0003DMG400001373, PGSC0003DMG400023211) and Phospholipase D (PGSC0003DMG400007848) were repressed in response to *Pba* SCRI1043*, Pbr* ICMP19477 and in the mock-inoculated control at 24 hpi.

Calcium influx

Consistent with the activation of the PI-PLC6 pathway and the proposed cytoplasmic Ca^{2+} influx during infection, expression of the gene encoding the calcium binding protein (PGSC0003DMG400023027) was up regulated in response to *Pba* SCRI1043 and *Pbr* ICMP19477 at 24 hpi. In addition, genes annotated as Calmodulin-binding proteins were significantly induced in response to *Pbr* ICMP19477 at 24 hpi (Table 4.2). This response appeared to occur in the mock-inoculated control as well (Table 4.2), although most of the associated genes showed only minor (non-significant) differential expression. Thus, calcium influx was probably associated with wounding, but exacerbated upon pathogen attack.

Respiratory Burst

Genes involved in the oxidative burst and in modulating ROS, such as those annotated as encoding cell wall peroxidase, peroxidase, glutaredoxin and thioredoxin II, were among the most highly DE in tubers in response to pectobacteria. For example, three out of five annotated putative peroxidase encoding genes (including cell wall peroxidase) were induced in response to *Pbr* ICMP19477 and *Pba* SCRI1043 at 24 hpi (Table 4.2). Genes encoding peroxidase (PGSC0003DMG400024967) were highly induced in response to mock-inoculation too. However, the magnitudes of the log2 fold changes observed in response to the two pathogens were consistently higher than in the mock-inoculated control (Table 4.2).

Cell wall modification

Genes related to cell wall modification, cell wall biosynthesis and cell wall degradation were significantly DE in response to pectobacteria and mock-inoculation. For example a gene annotated as cellulose synthase (*CslG*), involved in cellulose synthesis, was significantly induced only in response to *Pba* SCRI1043 and *Pbr* ICMP19477. In contrast, genes annotated as expansin, pectate lyase and pectinesterase involved in cell wall modification and cell wall degradation, were significantly down regulated in response to the pectobacteria at 24 hpi. Non-significant down regulation of these genes was also observed in response to mock-inoculation. Two out of five genes annotated as xyloglucan endotransglycosylase (PGSC0003DMG400021877, PGSC0003DMG400024755), involved in cell wall modification, were also significantly induced in response to pectobacteria and mock-inoculation at 6 and 24 hpi. These data suggested that systemic cell wall modifications occur in potato tubers upon exposure to wounding and to pathogen attack, although the latter causes the greatest response.

Table 4.2: A list of selected DEGs associated with early defence signalling in response to *Pbr* ICMP19477, *Pba* SCRI1043 and mock-inoculation across all sampling time.

Gene ID	Transcript Name	Mock-inoculated			*Pbr* ICMP19477			*Pba* SCRI1043		
		6 hpi	12 hpi	24 hpi	6 hpi	12 hpi	24 hpi	6 hpi	12 hpi	24 hpi
Phosphoinositol-phospholipase signalling pathway										
PGSC0003DMG400001373	Phospholipase C			-2			**-2.1**			**-2.2**
PGSC0003DMG400023211	Phospholipase C			-1.7			**-2.7**			**-2.4**
PGSC0003DMG400007848	Phospholipase D			-1.8			**-2.5**			**-2**
PGSC0003DMG400030360	PI-phospholipase C PLC6			1.6			**3.2**			**3.4**
PGSC0003DMG400014228	Inositol polyphosphate multikinase		1.2			**2.2**	**2.6**		**2.3**	**1.9**
PGSC0003DMG400024219	Inositol-1,4,5-triphosphate-5-phosphatase			1.7			**2.2**			**2.4**
Calcium signalling										
PGSC0003DMG400023027	Calcium-binding protein		1.7	**2.1**		**3.1**	**2.9**		1.8	**2.1**
PGSC0003DMG400026159	Calcium-binding protein (CCD1)						**2.3**			
PGSC0003DMG400001333	Calcium ion binding protein			1.4			**2.6**		1.5	
PGSC0003DMG400018102	Calmodulin-binding protein						**2.4**			1.6
PGSC0003DMG400015310	Calmodulin-binding protein						**2.0**			
Oxidative burst										
PGSC0003DMG400027614	Cell wall peroxidase					1.8			**3.9**	
PGSC0003DMG400015106	Cell wall peroxidase					1.4			**3.4**	
PGSC0003DMG400024967	Peroxidase			**2.4**			**4.5**			**3.2**
PGSC0003DMG402025083	Peroxidase						**2.1**			1.1
PGSC0003DMG400025084	Peroxidase 4						3			1.7
Cell wall modification										
PGSC0003DMG400024755	Xyloglucan endotransglucosylase 1	2.0			**3.2**			**4.2**		
PGSC0003DMG400021877	Xyloglucan endotransglycosylase			**2.7**			**2.9**			**2.3**
PGSC0003DMG400012852	Cellulose synthase (*CslG*)					**2.1**				**2.9**

PGSC0003DMG400009951	Expansin11			-1.8			**-2.3**		**-2.8**
PGSC0003DMG400004404	Expansin9			-1.6			**-2.4**		**-2.3**
PGSC0003DMG400029645	Pectate lyase			-1.5			**-2.6**		**-2.2**
PGSC0003DMG400012640	Pectate lyase P18			-1.5			**-3.7**	**-2.1**	**-2.6**
PGSC0003DMG400017929	Pectinesterase	-1.2	-1.8	-1.7			**-2.3**	**-2.1**	
PGSC0003DMG400003866	Xyloglucan endotransglucosylase	-1.2	-1.4	**-2.3**			**-2.8**		**-2.8**
PGSC0003DMG400000408	Xyloglucan endotransglucosylase-						**-2.3**	**-2.4**	**-2.1**
PGSC0003DMG400004109	Xyloglucan endotransglycosylase	**-2.7**	**-2.1**	**-2.5**	**-2.7**	**-2.4**	**-2.6**		**-2.4**

Note: The non-inoculated control was used to normalize DEGs. When a value is not present the data did not pass the quality control. The statistically significant DEGs are presented in **BOLD.** Numerical values represent the log2 fold change, positive values indicate the induced expression of the transcript when compared to the non-inoculated control and negative values indicate the repression of the transcript when compared to the non-inoculated control.

4.3.3.2 Hormonal modulation

Inoculation of potato tubers with *Pbr* ICMP19477 and *Pba* SCRI1043 resulted in the modulation of multiple plant hormone including the SA, JA, ET and gibberellic acid (GA) biosynthesis and signalling pathways. Differential expression of the pathways was observed primarily at 24 hpi (Table 4.3).

The ethylene-induced defence response

A number of genes related to ET biosynthesis were DE in response to *Pbr* ICMP19477, *Pba* SCRI1043 and mock-inoculation (Table 4.3). For example, multiple genes annotated as 1-amino-cyclopropane-1-carboxylate synthase (*ACS*) and 1-aminocyclopropane-1-carboxylate oxidase (*ACO*) were identified to DE in this analysis. Of these, three *ACS* genes and two *ACO* genes were up regulated in response to *Pbr* ICMP19477. Another gene encoding *ACS* (PGSC0003DMG400000193) was also significantly induced in response to *Pbr* ICMP19477 and *Pba* SCRI1043 at 24 hpi. Genes annotated as *ACO* were induced in response to mock-inoculation.

Although no significant differential expression of ethylene insensitive 3 (*EIN3*) and *EIN3*-like 2 (*EIL2*) were observed in response to either *Pbr* ICMP19477 or *Pba* SCRI1043, many genes involved in ET signalling including the ET receptor (PGSC0003DMG400017186), ET response factors (*ERF3*, *ERF4*, *ERF10*) and ET responsive TFs (*ERTF*, *ERTF4*) (PGSC0003DMG400017233, PGSC0003DMG400026821) were induced. In addition, a gene encoding *defensin* (PGSC0003DMG400015129; see JA-associated genes in Table 4.2), the host defence protein activated in response to necrotrophic pathogens via ET and JA signalling, was significantly down regulated in response to *Pba* SCRI1043 and non-significantly (-1.9) in response to *Pbr* ICMP19477. Expression of other plant defence-associated marker genes triggered by ET signalling including class 1 chitinase (PGSC0003DMG400010283) and endochitinase 2 (PGSC0003DMG400026854), were induced in response to both *Pbr* ICMP19477 and *Pba* SCRI1043 (Table 4.2). Expression of class 1 chitinase was also induced in response to mock-inoculation.

The jasmonic acid responsive defence response

Several genes involved in the early stages of JA biosynthesis and JA-responsive signal transduction were DE in response to *Pbr* ICMP19477 and *Pba* SCRI1043, although there did not appear to be the global induction of JA biosynthesis pathway that was expected (Table 4.3). Indeed, genes involved in the conversion of linolenic acid to JA, including allene oxide cyclase (*aoc*) and allene oxide synthase (*aos*), were not DE in response to pectobacteria or mock-inoculation. Furthermore, no significant differential expression of *JAR1* and *JMT* genes was observed in response to pectobacteria. *JAR1* and jasmonate O-methyltransferase genes are involved in the metabolism of JA to JA-Ile and MeJA (Staswick & Tiryaki, 2004). In contrast, a gene annotated as divinyl ether synthase (*des*) (PGSC0003DMG400025158), involved in the conversion of the 9-hydroxy linoleic and 9-hydroxy linolenic acid to colneleic and colnelenic acid, respectively, was up regulated in response to *Pbr* ICMP19477 and *Pba* SCRI1043 (Table 4.3) and non-significantly upon mock-inoculation at 24 hpi.

Genes involved in downstream JA signalling were DE in response to *Pba* SCRI1043 and *Pbr* ICMP19477, but not in response to the mock-inoculation (Table 4.3). For example, genes annotated as *MYC2* (PGSC0003DMG400012237) and jasmonate ZIM domain protein1 (*JAZ*) (PGSC0003DMG400002930) were significantly induced in response to *Pbr* ICMP19477 (Table 4.3). Though *MYC2* and *JAZ* were up regulated in response to *Pba* SCRI1043, the difference was not statically significant (p_{adj} =N/A).

The salicylic acid induced defence response

Genes related to the SA signalling pathways were DE in response to infection with both *Pba* SCRI1043 and *Pbr* ICMP19477 and wounding. Genes encoding glutaredoxin-C9 (*GRX480*) and thioredoxin II (TRXH5), were induced in *Pbr* ICMP19477 and *Pba* SCRI1043 across all sampling times (Table 4.3). Furthermore, several genes annotated as *PR1*, involved in the SA-dependent SAR response, were significantly up regulated in response to *Pbr* ICMP19477 at 24 hpi. Non-significant induction of *PR1* genes was also observed in response to *Pba* SCRI1043 and the mock-inoculated control at 24 hpi.

The gibberellic acid induced defence response

Genes annotated as involved in the later stages of the GA biosynthetic pathway, including GA20 oxidase (*GA20ox*), GA3 oxidase (*GA3ox*) and GA2oxidase (*GA2ox*), were DE in response to pectobacteria (Table 4.3). Of particular interest, three out of the five genes annotated as encoding *GA2ox* (involved in the metabolism of GA4 and GA1 (bioactive forms of GA) to GA34 and GA8, respectively), were significantly induced in response to *Pbr* ICMP19477 and *Pba* SCRI1043 at 6 and 12 hpi. Non-significant induction of these genes was also observed in response to mock-inoculation.

In addition, the gene encoding DELLA (negative regulator of GA) was induced in response to *Pbr* ICMP19477 and *Pba* SCRI1043 at 24 hpi. The Snakin 2 (*Sn-2*) cysteine rich peptide, which has antimicrobial properties, was also significantly up regulated in response to *Pbr* ICMP19477 and non-significantly in *Pba* SCRI1043 treated tubers. Furthermore, non-significant induction of Snakin 1 (*Sn-1*) (p_{adj} = N/A) was also observed in response to *Pbr* ICMP19477. No significant induction of these genes was observed in response to mock-inoculation (Table 4.3).

Table 4.3: A list of selected DEGs associated with hormone modulation in response to *Pbr* ICMP19477, *Pba* SCRI1043 and mock-inoculation across all sampling time.

Gene ID	Transcript Name	Mock-inoculated			*Pbr* ICMP19477			*Pba* SCRI1043		
		6 hpi	12 hpi	24 hpi	6 hpi	12 hpi	24 hpi	6 hpi	12 hpi	24 hpi
Ethylene biosynthesis and signalling										
PGSC0003DMG401026923	1-aminocyclopropane-1-carboxylate oxidase	**2.5**			**2.8**			1.4		
PGSC0003DMG400013894	1-aminocyclopropane-1-carboxylate oxidase 2						**2.7**			
PGSC0003DMG400012186	1-amino-cyclopropane-1-carboxylate synthase						**2**			
PGSC0003DMG400000193	1-aminocyclopropane-1-carboxylate synthase 2					**2.7**	**4**			**2.1**
PGSC0003DMG400005825	ACC synthase						**2.8**			1.5
PGSC0003DMG400010724	ERF transcription factor 4		1.3	1.3			**2.2**			1.4
PGSC0003DMG400017186	Ethylene receptor			1.1			**2.1**			1.9
PGSC0003DMG400014594	Ethylene response factor			1.8			**3.3**			**2.3**
PGSC0003DMG400010285	Ethylene response factor 10			**2.0**			**3.4**		**2.2**	**2.4**
PGSC0003DMG400022823	Ethylene response factor 3			1.6			**2.9**			**2**
PGSC0003DMG400017233	Ethylene-responsive transcription factor					**2.0**	**3.4**		**3.2**	
PGSC0003DMG400026821	Ethylene-responsive transcription factor 4						**2**			
PGSC0003DMG400029713	Putative ethylene responsive element binding protein 1		1.1			**2.2**	**2.2**		**2.0**	
PGSC0003DMG400017231	Transcription factor TSRF1			1.4			**3.2**			**3.5**
PGSC0003DMG400013402	Transcription factor TSRF1			**2**			**4.2**			3
PGSC0003DMG400026261	ATERF-2/ATERF2/ERF2			1.6			**3.5**		**2.3**	1.9
PGSC0003DMG400026260	AP2 domain class transcription factor			1.1			**2.8**		1.2	1.6
PGSC0003DMG400025282	AP2/ERF domain-containing transcription factor	1.4	1.6		**3.5**			**2.3**		
PGSC0003DMG400013608	AP2/ERF domain-containing transcription factor						**2.4**		1.2	1.6
PGSC0003DMG400010283	Class I chitinase			**2.7**			**6.4**			**3.7**
PGSC0003DMG400026854	Endochitinase 2				-1.2		**2.2**			1.5
PGSC0003DMG400011842	Chitinase				-1		**2.0**			
Jasmonic acid biosynthesis and signalling										

PGSC0003DMG400010859	Lipoxygenase						1.3				1.5
PGSC0003DMG400025158	Divinyl ether synthase			1.5		1.4	**3.6**		1.3		**2.1**
PGSC0003DMG400019881	JAR1										
PGSC0003DMG400003607	Jasmonate O-methyltransferase (*JMT*)										
PGSC0003DMG400012237	MYC2					**2.1**	**5.0**		1.1		2.5
PGSC0003DMG400002930	Jasmonate ZIM-domain protein 1					1	**2.5**		1.6		
PGSC0003DMG400015129	Defensin protein						-1.9				-2.0
Salicylic acid signalling											
PGSC0003DMG400019506	Thioredoxin II	**2.4**	**2.1**	1.5	**3**	**2.9**	**2.4**	**2.7**	**2.1**		**2.1**
PGSC0003DMG400009399	Glutaredoxin-C9	1.6	1.4	1.2	1.5	**2.2**	**3.1**	1			1.8
PGSC0003DMG400005109	PR-1						**2.4**				1.5
PGSC0003DMG400005110	PR1 protein						**2.8**				1.6
PGSC0003DMG400005111	PR1 protein			1.1			**2.7**				1.4
Gibberellic acid biosynthesis and signalling											
PGSC0003DMG400019211	DELLA protein GAI						**2.0**				1.2
PGSC0003DMG400024249	Gibberellin 20-oxidase-1						**-2.3**				-2.2
PGSC0003DMG400035710	Gibberellin 3beta-hydroxylase3						**-2.0**				-2.3
PGSC0003DMG400016516	Gibberellin 3-oxidase						**-2.4**				-1.8
PGSC0003DMG400027645	Gibberellin 2-oxidase	**2.5**			**3.2**	**2.6**		**2.1**			2.0
PGSC0003DMG400027631	Gibberellin 2-oxidase	1.6	1.1		**2.3**	**2.2**		1.8			1.5
PGSC0003DMG400002068	Gibberellin 2-oxidase 1			-1.6	-1.4		**-2.5**				-1.8
PGSC0003DMG400027632	Gibberellin 2-oxidase 2	1.4	1.3		**2.0**	**2.0**	**2.3**	**2.3**	1.7		1.9
PGSC0003DMG400021292	Gibberellin 2-oxidase 3						**-2.4**				-2.0
PGSC0003DMG400023235	Sn-2 protein						**6.1**		1.7		
PGSC0003DMG400042669	Sn-1 protein						5.2				

Note: The non-inoculated control was used to normalize DEGs. When a value is not present the data did not pass the quality control. The statistically DEGs are presented in **BOLD.** Numerical values represent the log2 fold change, positive values indicate the induced expression of the transcript when compared to the non-inoculated control and negative values indicate the repression of the transcript when compared to the non-inoculated control.

4.3.3.3 Transcriptional Factors

In addition to the ET responsive factors (section 4.3.3.2), a number of other TFs, including the *MYB* and *WRKY* TFs, were DE in response to *Pbr* ICMP19477 and/or *Pba* SCRI1043 (Table 4.4). In particular, genes annotated as *MYB108* (PGSC0003DMG400008761, PGSC0003DMG402004611, PGSC0003DMG400027157) were induced in response to both *Pba* SCRI1043 and *Pbr* ICMP19477, whereas *MYB78* (PGSC0003DMG400004610) and *MYB*-like 2 (PGSC0003DMG400027418), regulated by ABA and GA, respectively, were only induced in response to *Pbr* ICMP19477. Non-significant differential expression of these *MYB*-related TFs was observed in response to *Pba* SCRI1043 and mock-inoculation, however (Table 4.4). JA-induced *WRKY40* (PGSC0003DMG400019824) was also significantly induced in response to both *Pbr* ICMP19477 and *Pba* SCRI1043. Two genes annotated as *WRKY33* were also up regulated in response to *Pbr* ICMP19477, one of which (PGSC0003DMG400016769) was also induced (non-significantly) in response to *Pba* SCRI1043. This was consistent with the up regulation of *WRKY75* (PGSC0003DMG400021895). No significant differential expression of *WRKY* TFs was observed upon mock-inoculation.

Table 4.4: A list of selected DEGs predicted to encode transcription factors in response to *Pbr* ICMP19477, *Pba* SCRI1043 and mock-inoculation across all sampling time.

Gene ID	Transcript Name	Mock-inoculated			*Pbr* ICMP19477			*Pba* SCRI1043		
		6	**12**	**24**	**6**	**12**	**24**	**6**	**12**	**24**
Transcription Factor										
MYB transcription factors										
PGSC0003DMG400008761	R2R3 transcription factor MYB108 1	1.0					**2.6**			**2.3**
PGSC0003DMG402004611	MYB transcription factor (MYB108)			1.2			**2.6**		1.2	**2.1**
PGSC0003DMG400027157	R2R3 transcription factor MYB108 1			1.3			**3.1**			1.9
PGSC0003DMG400027418	GA-MYB-like2	1.5	1.3	1.3		**2.3**		1.7		1.2
PGSC0003DMG400004610	ABA-induced MYB transcription factor (MYB78)						**2.8**			1.1
WRKY Transcription factor										
PGSC0003DMG400021895	WRKY-type DNA binding protein (*WRKY75*)						**2.2**			1.8
PGSC0003DMG400019824	JA-induced WRKY protein (*WRKY40*)	1.7	1.6	1.7	1.8	**3.2**	**4.1**	1.6	**2.3**	**3.7**
PGSC0003DMG400011633	WRKY-type transcription factor (*WRKY33*)						**2.0**			
PGSC0003DMG400016769	Double WRKY type transcription factor (*WRKY33*)						**2.1**			1.9

Note: The non-inoculated control was used to normalize DEGs. When a value is not present the data did not pass the quality control. The statistically DEGs are presented in **BOLD.** Numerical values represent the log2 fold change, positive values indicate the induced expression of the transcript when compared to the non-inoculated control and negative values indicate the repression of the transcript when compared to the non-inoculated control.

4.3.3.4 Secondary Metabolism

A number of genes relating to the phenylpropanoid biosynthetic pathway including the hydroxyl cinnamoyl (PGSC0003DMG400018934), cinnamoyl–CoA reductase (PGSC0003DMG400010092, PGSC0003DMG400000521), hydroxycinnamoyl CoA:quinate hydroxycinnamoyl transferase (*HQT*) (PGSC0003DMG400011189) were significantly down regulated in response to *Pbr* ICMP19477 and *Pba* SCRI1043 (Table 4.5). Non-significant down-regulation of these genes was also observed in response to the mock-inoculated control. In contrast, genes annotated as involved in anthocyanin production were up regulated in all treatments, although the fold changes were greatest upon exposure to the pathogens.

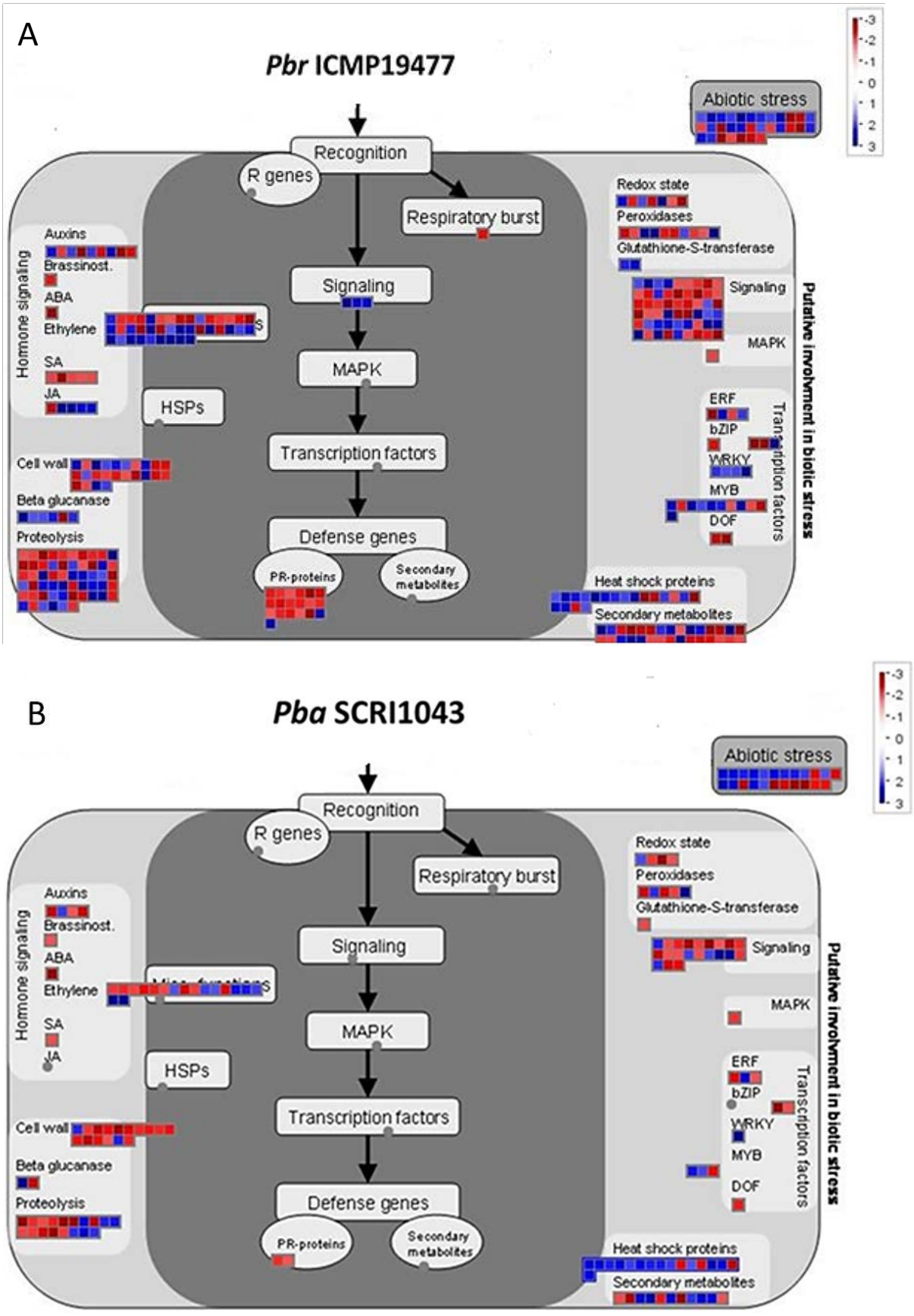
The biosynthetic pathways involved in the production of flavonoids were down regulated in all treatments (Table 4.5). For example, genes annotated as chalcone isomerase (PGSC0003DMG400011655), flavanone 3 beta-hydroxylase (PGSC0003DMG400003563), flavonol 4'-sulfotransferase (PGSC0003DMG400028350) and flavanone 3–hydroxylase (PGSC0003DMG404000594, PGSC0003DMG402000594, PGSC0003DMG401000594), were significantly down regulated in response to pectobacteria and non-significantly when mock-inoculated (Table 4.5). In contrast, terpenoid biosynthesis appeared to be induced, especially in potato tubers under pathogen attack. For example, two out of three genes annotated as sesquiterpene synthase were up regulated in response to infection (Table 4.5). In addition, genes annotated as epidermal germacrene C synthase (PGSC0003DMG400006694) and cembratrienol synthase 2a/ germacrene B synthase (PGSC0003DMG400011777), involved in germacrene biosynthesis, were significantly up regulated in response to pectobacteria. Germacrenes are bitter sesquiterpene olefins produced by higher plants during biotic stress, which have antimicrobial, antifeedant, and insecticidal properties (Adio, 2009). Induction of the terpenoid and germacrene biosynthetic pathways suggests these compounds are actively synthesised for defence against pectobacteria. Differential expression of the secondary metabolite biosynthesis pathway related to exogenous CFA is discussed in chapter 5, but have been provided here for comparative purposes.

Table 4.5: A list of selected DEGs predicted to encode secondary metabolites in response to *Pbr* ICMP19477, *Pba* SCRI1043, exogenous CFA and mock-inoculation across all sampling time.

Gene ID	Transcript ID	Mock-inoculation			*Pbr* ICMP19477			*Pba* SCRI1043			Purified CFA		
		6 hpi	12 hpi	24 hpi	6 hpi	12 hpi	24 hpi	6 hpi	12 hpi	24 hpi	6 hpi	12 hpi	24 hpi
Phenylpropanoids													
PGSC0003DMG400027180	Anthocyanin 5-aromatic acyltransferase			1.6	-1.0		**3.0**	-1.9		**2.5**			**3.3**
PGSC0003DMG400027181	Transferase family protein			1.9			**3.0**	-1.7	1.0	**2.6**			**3.9**
PGSC0003DMG400027127	Anthocyanin 5-aromatic acyltransferase			1.6		1.1	**2.5**			**2.2**			**2.9**
PGSC0003DMG400014466	4-coumarate--CoA ligase 2			-1.3			**-2.1**			-1.4			-1.1
PGSC0003DMG400025882	Caffeoyl-CoA O-methyltransferase 6	-1.4		-1.4	-1.2	-1.5	**-2.1**		-1.2	-1.7		-1.1	-1.5
PGSC0003DMG400007155	Anthranilate N-benzoyltransferase protein	-1.9	-1.6	-1.1	-1.9	-1.2	**-2.1**	-1.2		-1.6	-1.1	-1.5	-1.8
PGSC0003DMG400011189	HQT	-1.1			-1.5	-1.6	**-2.2**		-1.5	-1.6			-1.0
PGSC0003DMG400000560	Catechol O-methyltransferase			-1.4		-1.2	**-2.4**			-1.1			-1.3
PGSC0003DMG400031348	Transferase family protein	-1.1	-1.6	-1.6		-1.4	**-2.8**			-1.7			-1.5
PGSC0003DMG400018934	Anthranilate N-hydroxycinnamoyl	-1.3	-1.4	-1.6	-1.4	**-2.9**	**-3.5**	-1.4	-1.9	**-3.1**		-1.7	**-2.3**
PGSC0003DMG400014959	Acyltransferase						1.8			1.8			2.3
PGSC0003DMG400025219	Acyltransferase 2	-1.7	-1.5	-1.3	-1.8	**-2.3**	-1.1	-1.6	-1.5	-1.9	-1.5	-1.9	**-2.0**
PGSC0003DMG400010092	Cinnamoyl-CoA reductase		-1.1	-1.1		-1.2	**-2.1**			-1.0		-1.1	-1.5
PGSC0003DMG400000521	Cinnamoyl-CoA reductase	**-2.1**	-1.9	-1.7	-1.8	**-2.3**	**-2.2**	-1.2	-1.1	-2.3	-1.1	-1.5	**-2.1**
Flavonoids													
PGSC0003DMG400025098	All-trans-retinol 13,14-reductase		-1.4	-1.4		-1.4	**-2.5**		-1.1	**-2.1**		-1.3	-1.5
PGSC0003DMG400011655	Chalcone isomerase	-1.6	-1.6	-1.9		**-2.1**	-1.8			-1.8		-1.1	-1.9
PGSC0003DMG400003563	Flavanone 3 beta-hydroxylase	-1.5	-1.2	-1.9	-1.7	-1.5	**-2.1**	-1.0		-1.6		-1.0	-1.8
PGSC0003DMG400028350	Flavonol 4'-sulfotransferase			-1.4		**-2.4**	**-4.0**			**-3.7**		-1.0	-1.8
PGSC0003DMG404000594	Flavonol synthase			-1.2			**-2.2**			-2.0			-2.0
PGSC0003DMG402000594	Flavonol synthase			-1.6			**-2.3**			-1.6			-1.7
PGSC0003DMG401000594	Flavonol synthase	-1.0	-1.1	**-2.1**		-1.6	**-4.3**			**-2.6**		-1.1	-2.7
PGSC0003DMG401009033	MYB transcription factor 12			**-2.1**		**-2.6**	**-2.7**		**-2.3**	**-2.4**		**-2.4**	**-2.4**

Terpenoids												
PGSC0003DMG400031559	Sesquiterpene synthase 2			1.4	-1.2	1.1	**3.1**	-1.3		**2.8**		**3.3**
PGSC0003DMG400011777	Cembratrienol synthase 2a			1.5	-1.9		**2.7**	**-2.3**		**2.6**		**2.8**
PGSC0003DMG400006694	Epidermal germacrene C synthase			1.0		1.0	**2.5**			1.6		**2.5**
PGSC0003DMG400017069	Beta-amyrin synthase			-1.6		-1.6	**-2.0**		-1.0	-1.1	-1.0	-1.9
PGSC0003DMG400010585	Sesquiterpene synthase		-1.5	-1.9		-2.0	**-3.5**	-1.0	-1.2	**-2.3**	-1.6	**-2.5**
PGSC0003DMG400020680	Sesquiterpene synthase (TPS13)	1.3				**2.1**	1.4					

Note: The non-inoculated control was used to normalize DEGs. When a value is not present the data did not pass the quality control. The statistically DEGs are presented in **BOLD**. Numerical values represent the log2 fold change, positive values indicate the induced expression of the transcript when compared to the non-inoculated control and negative values indicate the repression of the transcript when compared to the non-inoculated control. Differential expression relating to exogenous CFA is provided here for comparative purposes, but are discussed in more detail in Chapter 5.


A
Pbr ICMP19477
Abiotic stress
Recognition
R genes
Respiratory burst
Signaling
MAPK
Transcription factors
Defense genes
PR-proteins
Secondary metabolites
HSPs
Hormone signaling
Auxins
Brassinost.
ABA
Ethylene
SA
JA
Cell wall
Beta glucanase
Proteolysis
Redox state
Peroxidases
Glutathione-S-transferase
Signaling
MAPK
ERF
bZIP
WRKY
MYB
DOF
Transcription factors
Heat shock proteins
Secondary metabolites
Putative involvment in biotic stress
B
Pba SCRI1043
Abiotic stress
Recognition
R genes
Respiratory burst
Signaling
MAPK
Transcription factors
Defense genes
PR-proteins
Secondary metabolites
HSPs
Hormone signaling
Auxins
Brassinost.
ABA
Ethylene
SA
JA
Cell wall
Beta glucanase
Proteolysis
Redox state
Peroxidases
Glutathione-S-transferase
Signaling
MAPK
ERF
bZIP
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Heat shock proteins
Secondary metabolites
Putative involvment in biotic stress

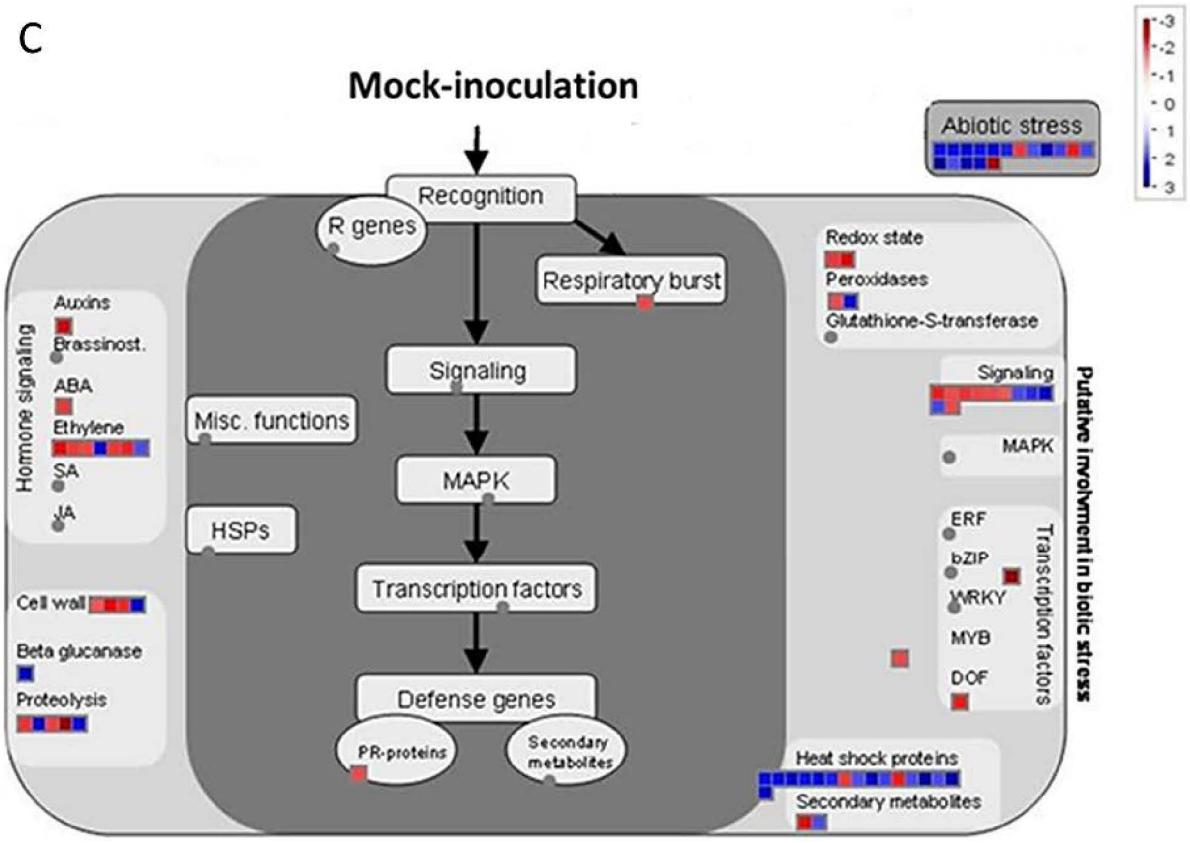


Figure 4.3: DEGs related to potato defence observed in response to (A) *Pbr* ICMP19477, (B) *Pba* SCRI1043 and (C) mock-inoculation at 24 hpi.
Potato Mapping (Stub_PGSC_DM_v3.4) and differential expression data obtained by DESeq2 analysis were used for MapMan analysis. Genes up and down regulated are visualised in blue and red, respectively.

4.3.4 Differential expression in response to *Pba* SCRI1043 and *Pbr* ICMP19477 when normalised with Mock-inoculation control

To identify the genes differentially expressed only in response to *Pectobacterium* infections, pairwise comparisons were conducted using the mapped reads from mock-inoculated control tubers and tubers treated with bacteria at each time point. Log2 fold change ≥ ±2 and $p_{adj} \leq 0.05$ were used as cut-offs to identify DEGs in response to *Pectobacterium* infections. In these comparisons, the number of DEGs identified (Appendix B.5) was significantly lower than that observed when normalised with the non-inoculated control; a total of 60 and 124 genes differentially expressed in response to *Pbr* ICMP19477 and *Pba* SCRI1043, respectively. Previously, it has been suggested that mechanical damage and cell death caused by pathogen infection result in differential expression of a common set of genes (Boller & Felix, 2009; Dangl

& Jones, 2001). In this study, DE of a core set of genes was evident in the log fold changes in response to the mock-inoculation control and bacterial infection when normalised with the non-inoculated control (section 4.3.3). The magnitude of differential expression observed in response to bacterial infection was significantly higher than after wounding, however. The similar responses of the core set of DE genes to wounding and pathogen attack led to pairwise comparisons using the mock-inoculated control producing lower log fold changes in response to bacterial infection. These lower log fold changes were not statistically significant, meaning that normalisation with the mock-inoculated control lacked the power to detect the greater defence response induced by the tuber against *Pectobacterium*. As the main aim of this study was to identify the early responses of potato tuber to *Pectobacterium* infection, normalising with non-inoculated control rather than the mock-inoculated control was considered be the most appropriate approach.

4.3.5 Metabolic Profiling of potato tubers in response to *Pectobacterium*

As the transcriptomic profiling identified an extensive modulation of secondary metabolism, the total phenolic compounds produced in response to pectobacteria was quantified using a microplate assay with a gallic acid (GAE) standard curve (section 2.3.1). The metabolites DE in response to these treatments were then profiled using LC-MS.

Changes in phenolic concentrations in potato tubers treated with *Pba* SCRI0143, *Pbr* ICMP19477 or after mock-inoculation were quantified at 6, 12 and 24 hpi by estimating the mean of the total phenolics in tubers after each treatment. The data from this analysis is summarised in Figure 4.4.

Results from this analysis indicated significant interaction between time and treatment ($p = 0.023$). When compared to the total phenolic content in the non-inoculated control, the concentration was consistently lower at 6 hpi. Furthermore, the total phenolic content in tubers in response to all treatments increased from 6 to 12 hpi. An increase in the total phenolics was also observed between 12 and 24 hpi in *Pba* SCRI1043 and *Pbr* ICMP19477 treated tubers, whereas the observed total phenolic concentration decreased in mock-

inoculated control. The results from this assay confirmed the influence of wounding and pathogen attack on total phenolic compounds in potato tubers.

In addition to the microplate assay, the phenolic compounds were also profiled using LC-MS analysis. Citric acid and the alkaloids α-solanine and α-chaconine two know phenolic compounds in potato tuber were used as reference. The concentration of these compounds showed no correlation with treatment, and were variable across replicates as expected in tuber flesh (Nigel Joyce, personal communication). Initial analysis identified a number of compounds whose presence/quantity correlated across treatments, however these were rejected because of their poor resolution on the applied phenolic analysis column. When compared with the mock-inoculated control, two unknown compounds with m/z 294, m/z 477 and retention time of 3.48 and 13.12 min, respectively were up regulated across treatments. The induction of these compounds was significant at 12 hpi (data not shown). However, the comparison of these compounds against the metabolic database failed to provide any conclusive results. Hence, further purification and chromatogram analysis is required to characterise these two compounds. Furthermore, due to the lower number of replicates in each treatment the data obtained from this study only provides suggestive trends, and hence the experiment will need be repeated with statistically acceptable replicates for further confirmation.

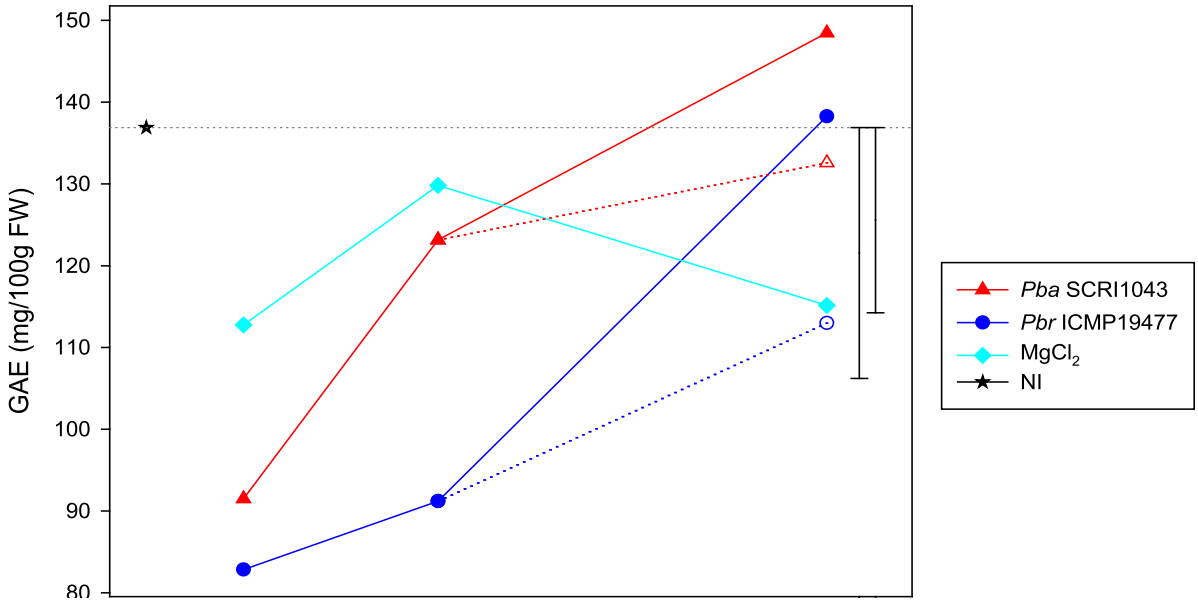


Figure 4.4: Estimated mean GAE in potato tubers ('Summer Delight') treated with *Pba* SCRI1043, *Pbr* ICMP19477, and mock-inoculation at 6, 12 and 24 hpi.

The mean GAE observed in the non-inoculated controls is represented by NI. The error bars represents the 5% least significant difference.

4.4 Discussion

4.4.1 The intensity of the transcriptional response in potato is determined by the aggressiveness of the SRE

In this chapter, having previously identified DESEq2 as an appropriate sotfware package for generating differential expression data from Illumina HiSeq RNA sequencing, DESeq2 was used to identify the global systemic transcriptional response of potato tubers ('Summer Delight') to infection by *Pbr* ICMP19477 and *Pba* SCRI1043. This was the first transcriptome-wide study focusing on the transcriptional response of potato tubers to enterobacterial phytopathogens, which are globally important to potato production due to their capacity to cause soft rot and blackleg disease (Perombelon, 1987, 1992, 2002).

Previous disease-related transcriptome studies in potato have focused on the response of the potato stem to *Pbr* (Kwenda et al., Unpublished) or have examined the interaction of potato tubers with a very different potato pathogen, *P. infestans* (Ali et al., 2014; Gao & Bradeen, 2016; Gao et al., 2013; Massa et al., 2011; Yogendra & Kushalappa, 2016). The former, although not yet published, identified the differences in the transcriptional responses of susceptible and tolerant cultivars to *Pbr*. However, the study provided no opportunity to understand the influence of virulence or aggressiveness, as it only used one bacterial strain. Furthermore, it did not enable the truest host-pathogen interaction, which occurs at the point of pathogen entry, to be examined; namely in the tubers where the pathogen gains entry through the lenticels or wounds (Perombelon, 1992, 2002; Perombelon & Hyman, 1989; Perombelon & Kelman, 1980). Studying the response in the tubers would seem particularly important, especially as studies in *Arabidopsis* have noted organ-specific disease resistance in response to isolates of *Hyaloperonospora arabidopsidis* (Hermanns et al., 2003). Similarly, studies in Maize, on the induced resistance in above-ground and below-ground organs in response to the fungal pathogen *Colletotrichum graminicola*, observed more rapid and induced expression of defence-related genes and higher levels of antimicrobial flavonoids in roots than in leaves. (Balmer et al., 2013). Recent studies in potato on organ specific defence mechanisms against the late blight pathogen *P. infestans* have also confirmed the difference in the cell wall metabolism and plant hormone-mediated defences between foliage and tubers (Gao & Bradeen, 2016), confirming the existence of tissue/organ-specific defence mechanisms in potato

In total, a core set of 319 genes were identified as DE in response to both *Pbr* ICMP19377 and *Pba* SCRI1043. These genes appeared to represent the core set of DEGs in tubers in a susceptible interaction with pectobacteria, irrespective of the aggressiveness of the pathogen. The total number of DEGs upon infection with both *Pba* SCRI1043 and *Pbr* ICMP19477 was highest at 24 hpi. Furthermore, a steady increase was observed in the number of genes down regulated in response to these pathogens. The relatively lengthy time required by the host to mount a significant transcriptional response and the subsequent increase in the number of down regulated genes within the period is characteristic of a susceptible host-pathogen interaction.

In addition to the core set of DEGs, another 937 genes were DE only in response to *Pbr* ICMP19477. This number was significantly greater than the number of genes DE only in response to *Pba* SCRI1043, suggesting that the aggressiveness of the strain was important in determining the response of the host. Given that the fold change was also greater in the DEGs in response to *Pbr* ICMP19477, the intensity as well as the type of host response would seem to be determined by the aggressiveness of the pathogen. This was supported by the plethora of DEGs (in response to *Pbr* ICMP19477) that were also non-significantly DE in response to *Pba* SCRI1043.

4.4.2 Pathogen and effector triggered defence signalling pathways appear to be triggered in response to pectobacteria

4.4.2.1 Early response to pectobacteria

Plants possess various PRRs, which are involved in the perception of pathogen-related molecular signatures (PAMPs) and damage signals elicited by plants during mechanical damage and pathogen-mediated plant damage (DAMPs). Perception of PAMPs/DAMPs by PRRs results in the activation of the PTI-triggered immune response (Boller & Felix, 2009; Dodds & Rathjen, 2010; Jones & Dangl, 2006). In this study, several genes annotated as encoding receptor protein kinases (RPKs) were DE in response to *Pbr* ICMP19477, *Pba* SCRI1043 and mock-inoculation (Table 4.1). Like many genes, however, the induction of the PRRs genes was greatest in response to *Pbr* ICMP19477 and least significant in *Pba* SCRI1043 treated tubers and mock-inoculated controls. Given that *Pbr* ICMP19477 is an aggressive pathogen, it is probable that the induction of these genes was associated with the perception of PAMP/DAMP-mediated signals derived from wounding as well as tissue maceration by the SRE. Studies to understand the potato response to culture filtrates of *Pcc* have observed the induction of receptor-like kinases (RLKs) in host tissue (Montesano et al., 2001). Furthermore, the induction of these RLK genes was observed at the site of inoculation as early as 1 hpi, while their induction in systemic/ distal tissues was low and delayed. These genes were also transiently induced in response to OGs (Montesano et al., 2001). Taken together, the observed induction of these genes and the previous data on *Pcc* suggest a role for RPKs in

PAMP/DAMP perception and signalling in potato tubers. Given the lack of annotation of these genes in potato, further sequence similarity searches and structural analyses would be required to confirm the relatedness of these receptors to the previously documented RPKs. Functional analysis would also be required, to confirm PAMPs and DAMPs perceptions by these RPKs.

Genes annotated as encoding NBS-LRRs were DE in tubers in response to infection with pectobacteria. NBS-LRRs are predicted to be associated with ETI (Dodds & Rathjen, 2010; Jones & Dangl, 2006), of particular interest was the significant but specific induction of several genes in response to *Pbr* ICMP19477 (although non-significant induction was observed in response to *Pba* SCRI1043) with homology to genes producing Avr9/C9-related proteins and kinase. Previous studies in tomato and tobacco have confirmed the induced expression of *ACIK1* upon elicitation by *Avr9* and by wounding (Rowland et al., 2005). Furthermore, silencing of *ACIK1* resulted in a reduction in the C9-mediated HR and resistance to *C. fulvum*, suggesting an important role for *ACIK1* in disease resistance to this pathogen (Rowland et al., 2005). The race-specificity of *ACIK1* and the induction of homologues in potato tubers in response to *Pbr* ICMP19477 would imply that 'Summer Delight' is able to elicit an ETI response to this pathogen. This is contradicted by the lack of known effectors in *Pectobacterium* spp., however, as DspE is the only known effector in pectobacteria (Kim et al., 2011; Kim et al., 2009). It must also be remembered that a successful ETI triggered by these potential *R* genes would elicit cell death and a HR, which was not observed in pathogenicity assays. Thus, even though it is tantalising to predict ETI, the identity of these genes and their function remain obscure and require further scrutiny.

The transduction of the extracellular and intracellular signals perceived by the LRR-RPKs and NBS-LRRs is mediated by calcium influx and an oxidative burst, which transfer signals to downstream signalling components including hormone-mediated defence responses, transcriptional reprogramming and secondary metabolite biosynthesis. Several genes predicted to be involved in stress-mediated signalling were DE in response to *Pbr* ICMP19477, *Pba* SCRI1043 and mock-inoculation (Table 4.2). The differential expression of these genes in response to mock-inoculation in particular was suggestive of these pathways being involved in the early DAMP-mediated PTI response in potato. PI-PLC signalling has been identified to

play a central role in the cellular defence response in mammals and in plants, and has been associated with both PTI- and ETI-mediated defence responses (Munnik, 2014). Studies in tomato, have reported that fully functional *SIPLC6* and *SIPLC4* genes, encoding key players in PI-PLC signalling, are required for the HR response induced by the effectors of *P. syringae*, (*Pto*) and *C. fulvum* (*Cf-4*), respectively (Vossen et al., 2010). In this study, the enzymes involved in the PI-PLC signalling pathway, including PI-PLC6, Inositol-1,4,5-triphosphate-5-phosphatase and *InsPK* were significantly up regulated at 24 hpi in response to both *Pbr* ICMP19477 and *Pba* SCRI1043. Furthermore, other genes relating to PLC including PLD, gene involved in PA production were significantly repressed in these treatments (Table 4.2). In *Arabidopsis*, recognition of the type III effectors *AvrRPM1* and *AvrRP2* from *P. syringae* resulted in biphasic accumulation of PA, involving the activation of the PLC followed by the subsequent activation of PLD (Andersson et al., 2006). Though the activation of the PLD resulted in a higher magnitude of PA accumulation, the influx in Ca^{2+} ions was observed only in response to the PLC activity. Furthermore, inhibition of the IP3 and IP6, two end products of the PI-PLC pathway resulted in the reduced cytosolic Ca^{2+} influx associated with DAMPs and PAMPs (Kwaaitaal et al., 2011; Lecourieux et al., 2006). These data placed the defence-mediated Ca^{2+} influx downstream of PLC activation and upstream of PLD activation (Andersson et al., 2006). Taken together, the results from this study suggest that the PI-PLC6-dependent and PLD-independent signalling pathway is essential for the early defence against wounding and pectobacteria infection.

Ca^{2+} influx is perceived by the calcium binding proteins such as *CaM*, *CDPK* and calcineuirn B-like proteins (*CBL*) (Lecourieux et al., 2006; Rudd & Franklin-Tong, 1999). Studies in tomato have observed accumulation of *CaM* mRNA and CaM protein in response to wounding (Bergey & Ryan, 1999). TMV infection in transgenic tobacco plants overexpressing *CaM4* and *CaM5* also resulted in spontaneous lesions and expression of SA-independent SAR-related genes. Furthermore, these plants showed enhanced disease resistance to a wide range of pathogens, suggesting CaM proteins may be involved in the SA-independent SAR response (Do Heo et al., 1999; Lecourieux et al., 2006). Studies to understand the defence response of transgenic *Arabidopsis "defence, no death"(dnd)* to *Pcc,* observed *CaM*-dependent defence responses, where treatment with W-7 and chlorpromazine, two CaM antagonists triggered induced cell death and disease development suggesting that *CaM*-mediated defence responses are crucial

for plant defence against pectobacteria (AHN, 2007). Altogether the results observed in this study suggest that PI-PLC signalling or PAMPs/DAMPs-mediated Ca^{2+} influx is essential for the early defence response against pectobacteria and wounding, and is speculated that it may play a central role in regulating downstream defence signalling in potato tubers.

4.4.2.2 Hormonal cross talk

Phytohormones play a central role in modulating the plant immune signalling network (Pieterse et al., 2012). In plants, JA, ET and SA have been identified for some time to modulate the plant defence response to pathogen attack (Pieterse et al., 2012). In contrast, other phytohormones including ABA, GA, AU and brassinosteroids have been described largely as plant growth regulators (Pieterse et al., 2012; Robert-Seilaniantz et al., 2011). Recent studies, however, have confirmed these growth hormones as key regulators of the plant defence, which maintain the balance between plant growth and development and responses to pests and pathogens (Pieterse et al., 2012; Robert-Seilaniantz et al., 2011; Yang et al., 2012). In addition, the antagonistic and synergistic interactions between phytohormones have been clearly shown to enhance the complexity of the plant immune defence response. In this study, differential expression was observed in a variety of hormone pathways in response to inoculation with pectobacteria including ET, JA, SA, and GA-mediated signalling pathway.

Changes in cellular redox play an important role in modulating plant immune responses including the hormone-mediated defence responses (Foyer & Noctor, 2011; Pieterse et al., 2012; Spoel & Loake, 2011). Glutaredoxin-C9 and Thioredoxin II are the central players in redox regulation and are involved in SA-associated defence (Meyer et al., 2012; Tada et al., 2008). In this study, genes annotated as encoding *TRXH5* and *GRXC9* were induced in response to both pathogen infection and wounding. For example, in SA-induced cells thioredoxin II enables monomerization of NPR1, resulting in its interaction with the TGA TFs to activate *PR1* (Spoel & Loake, 2011). Furthermore, over-expression of *GRXC9* in *Arabidopsis* resulted in the down regulation of the JA-responsive *PDF1.2* gene (encoding defensin), suggesting a strong antagonist interaction between the SA and JA signalling pathways (Meyer et al., 2012) Later, *GRXC9* knockout studies failed to observe induction of the JA pathway, however, suggesting

that *GRXC9* was not essential for the SA-JA antagonistic interaction (Herrera-Vásquez, Carvallo, et al., 2015).

Studies in *Arabidopsis* to understand the host response to *D. chrysanthemi* observed induced expression of *PR1* at 24 hpi (Fagard et al., 2007). However, further investigation to understand the importance of the SA-mediated defence response to *D*. *chrysanthemi* in transgenic *sid2* mutants (impaired of SA production), failed to observe significant disease development. These results suggested that the SA-mediated defence response may be dispensable for efficient defence against *D*. *chrysanthemi* invasion (Fagard et al., 2007). Consistent with the lack of differential expression in the SA biosynthetic pathway and the up regulation of the *TRXH5* and *GRXC9* genes in potato tubers in response to *Pectobacterium* infection in this study, the observed induction of *PR1* is probably a result of a redox-mediated SA defence response to this necrotrophic/hemibiotrophic pathogen. However, the role of *TRXH5* and *GRXC9* in the SA pathway in potato has not been confirmed and thus the mechanism by which redox plays a role in the defence against SRE and wounding remains unknown.

Genes relating to ET biosynthesis were significantly induced in response to pectobacteria and to a lesser extent in the mock-inoculated controls. For example, two out of three genes annotated as *aco*, involved in the ET biosynthesis, were significantly induced in response to wounding and *Pbr* ICMP19477 and to a lesser extent in *Pba* SCRI1043. ACOs are encoded by multigene families and previous studies have identified that the ACO encoding genes were DE in response to different biotic and abiotic stresses (Broekaert et al., 2006). Induction of the different *aco* genes and their varied expression profiles across the treatments confirmed the differential expression of these genes in response to different biotic and abiotic stresses. Generic induction of ET biosynthesis in potato tuber during infection with pectobacteria and wounding suggested this response was associated with DAMP-mediated defence signalling.

Consistent with the induced expression of ET biosynthetic genes, genes related to downstream ET signalling were also up regulated in response to the both *Pbr* ICMP19477 and *Pba* SCRI1043. In particular, genes annotated as encoding TSRF1 were significantly induced in response to both pectobacteria. Orthologues of these genes in *Arabidopsis* are annotated as *ERF1*. *ERF1* acts downstream of *EIN3* and *EIL*. *ERF1* belongs to a large family of ET response

element binding proteins (EREBPs) that bind to the GCC-box, a promoter motif present in defence genes induced by pathogens and ET (Guo & Ecker, 2004; Solano et al., 1998). In *Arabidopsis*, *ERF1* is also involved in the regulation of the JA-mediated defence response, where pathogen-induced JA/JA-Ile biosynthesis results in the activation of the *ERF1* branch of JA mediated signalling, resulting in the coordinate induction of plant defensin (*PDF1.2)*. In this study, the down regulation of plant defensin suggested that the induced expression of *ERF1* and the ET receptors and the TFs is related to the ET-dependent and JA independent defence response.

In this study, the genes encoding *lox* involved in the biosynthesis of the JA precursor's 13-hydroperoxy and 9-hydroperoxy linoleic and linolenic acid were induced in response to the *Pectobacterium* spp. However, differential expression of genes involved in the conversion of the 13-hydroperoxy linoleic acid to JA, including *aos* and *aoc,* were not DE. Interestingly, recent studies in potato on the tuber and foliage response has also failed to observe the induction of the JA-mediated defence response against *P. infestans* (Gao & Bradeen, 2016). Instead, *des*, involved in metabolism of9-hydroperoxy linoleic and linolenic acid to colneleic and colnelenic acid (Figure 4.5) was induced in response to *Pectobacterium* spp. Furthermore, this gene was also induced to a lesser extent in the mock-inoculated controls, suggesting the involvement of colneleic and colnelenic acid in both the wound and pathogen-mediated defence response in tubers. Similar results were observed in potato leaves and tobacco roots during infection with *P. infestans* (Weber et al., 1999), *P. syringae* (Stumpe et al., 2001) and *P. parasitica* (Fammartino et al., 2007). *P. infestans* infection in potato leaves resulted in the expression of colneleic and colnelenic acid, further the expression was highly induced in the resistant potato 'Matilda' when compared to the susceptible 'Bintje', which suggested antimicrobial properties for colneleic and colnelenic acid (Weber et al., 1999). Further studies to characterize *des* in tobacco have confirmed the role of colneleic and colnelenic acid in enhanced resistance to *P. parasitica* (Fammartino et al., 2007). In particular, transgenic tobacco plants with antisense *Lox1* genes (*9-Lox*) impaired in DES and subsequently the colneleic and colnelenic acid showed enhanced susceptibility to the pathogen. These results confirmed the role of a DES related JA pathway in modulating the defence response during tobacco-*P. parasitica* interactions (Fammartino et al., 2007).

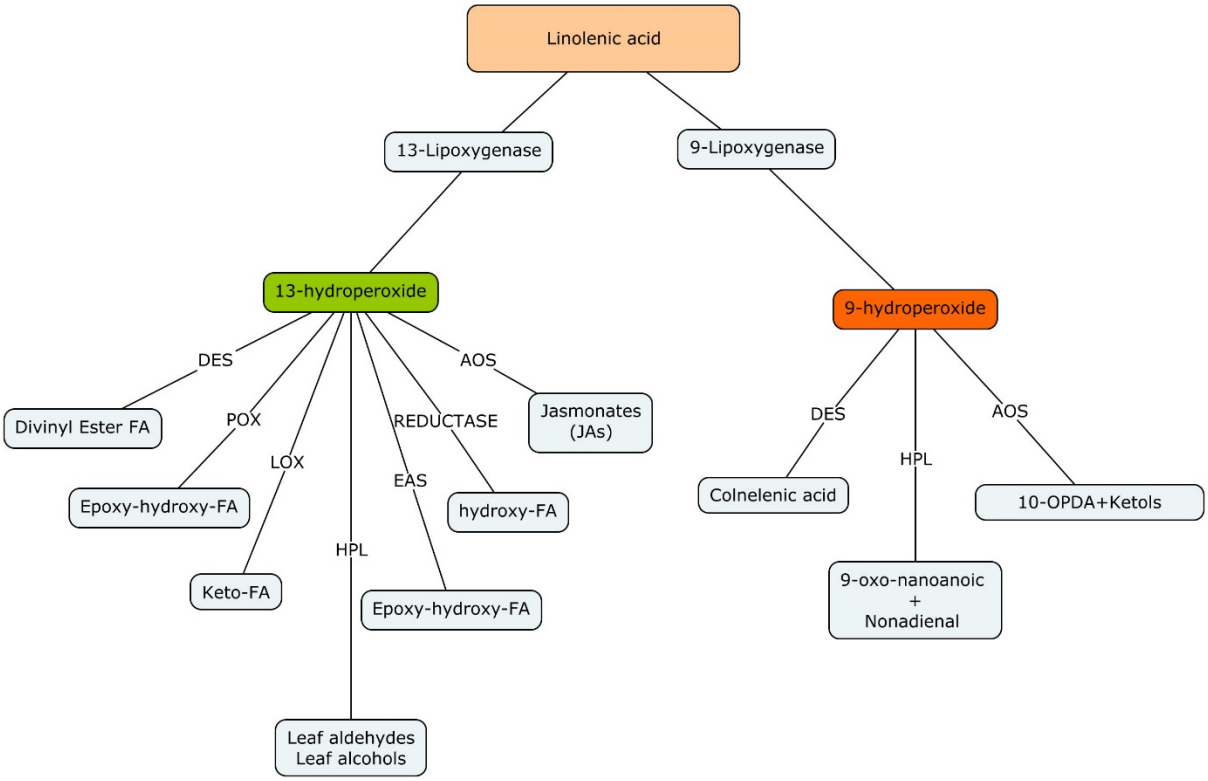


Figure 4.5: The 13-lipoxigenase and 9-lipoxygenase mediated metabolism of linolenic acid.

Studies in *Arabidopsis*, tomato and tobacco suggest that the JA responsive defence response is essential for the plant's defence against necrotrophic pathogens, some phloem feeding insects and chewing herbivores (Arimura et al., 2011; Fürstenberg-Hägg et al., 2013; Yan & Xie, 2015). JA mediated defence signalling in plants, requires the plant hormone JA to be sequentially synthesized and conjugated to amino acids such as isoleucine (JA-Ile) via JA-amino acid synthase (*JAR1*) (Staswick & Tiryaki, 2004; Staswick et al., 2002). In addition to the lack of differential expression in the JA biosynthetic pathway, in this study *JAR1* was not DE in response to the pathogen or wounding. Interestingly, however, *MYC2* and its repressor JAZ proteins involved in downstream JA signalling were induced in response to *Pbr* ICMP19477 and to a lesser extent in *Pba* SCRI1043 during 12 and 24 hpi. JAZ proteins act as the transcriptional repressors of JA signalling by binding to the positive transcriptional regulator *MYC2* (Fernandez-Calvo et al., 2011; Niu et al., 2011). During pathogen induced JA stimulation, the F box protein CORONATINE INSENSITIVE (COI1) together with JAZ proteins act as the co-receptor of JA-Ile (Figure 4.6). Binding of the biologically active JA-Ile to the COI1 domain leads to ubiquitination by the E3 ubiquitin-ligase SKP1-Cullin F-box complex (SCF^{COI1}) and degradation of the JAZ repressor protein and activation of *MYC2* (Memelink, 2009). In

Arabidopsis, two branches of the JA responsive signalling pathway have been reported, the *MYC2* branch and *ERF* branch (Memelink, 2009; Zhu et al., 2011). The activation of the *MYC2* branch of the JA responsive signalling results in the induced expression of VSP1 and *JAZ* and was identified to be mutually antagonistic with the *ERF* branch of the JA-mediated defence response (Figure 4.5). Thus, activation of the *MYC2* results in the repression of *PDF1.2*, which encodes the defensin required for defence against necrotrophic pathogens (Pieterse et al., 2012). In this study, the gene encoding defensin was significantly down regulated in response to *Pba* SCRI1043, and non-significantly in response to *Pbr* ICMP19477.

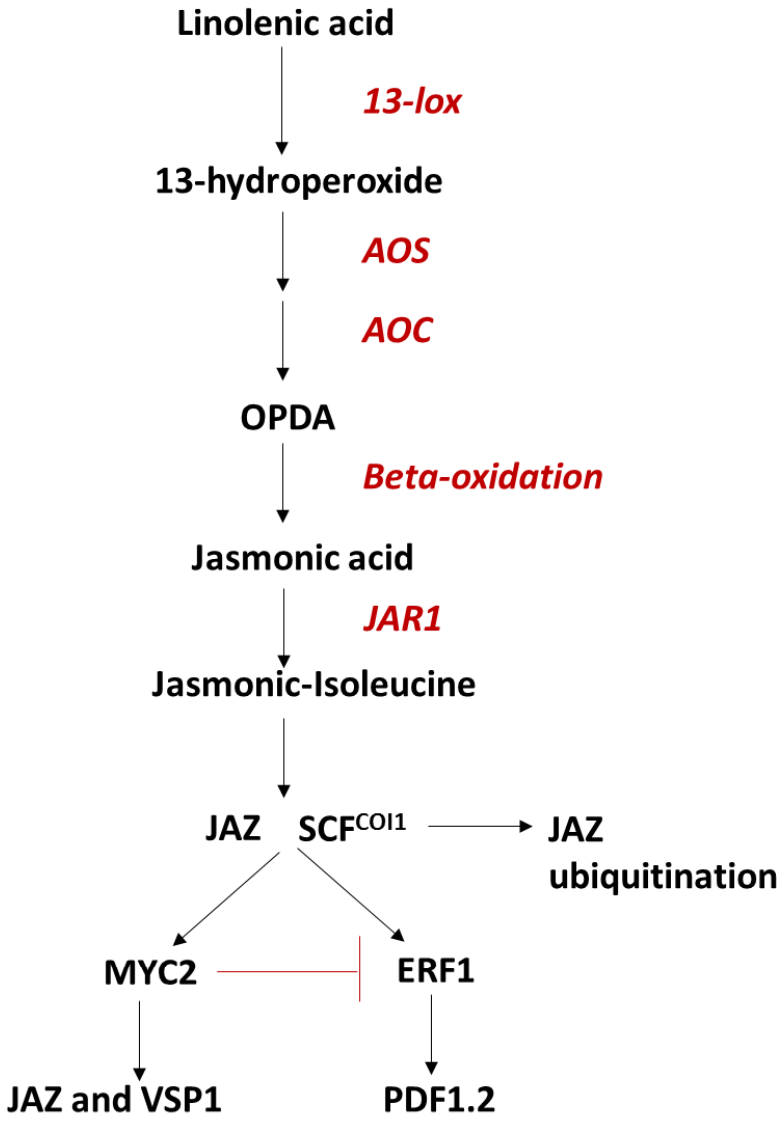


Figure 4.6: Diagrammatic representation of jasmonic acid biosynthesis and signalling pathway in Arabidopsis.

In *Arabidopsis*, induction of the *ERF* branch of JA defence response is required for defence response against necrotrophic pathogens, while the *MYC2* branch is required for defence against the herbivores and for the priming the distal organs against future herbivores attack (Kazan & Manners, 2012). In *Arabidopsis*, the expression of the *ERF* branch of JA responsive genes including *ORA59* resulted in the suppression of the *MYC2* branch and further enhanced the susceptibility of the plants to *Pieris rapae* larvae (Schweizer et al., 2013). Furthermore, mutation in the *MYC2* resulted in the *Arabidopsis* plants being more susceptible to insect stress (Dombrecht et al., 2007). Induced expression of the genes annotated as encoding *MYC2* and the *JAZ* in pectobacteria-infected tubers contradicts the lack of differential expression of genes related to JA biosynthesis and its conjugation to an amino acid. Based on the previous studies on plant-microbe interactions (Sheard et al., 2010; Smith et al., 2009), the apparent induction of COI1-JAZ-MYC2-mediated defence in response to pectobacteria seems an inappropriate defence response activated against this pathogen. The questions remains why this takes place?

In addition to JA, SA and ET mediated defence responses, genes related to GA biosynthesis and signalling were DE in response to pectobacteria and mock-inoculation. Interestingly, induction of genes involved in conversion of bioactive GA to non-active GA and the induction of DELLA, the repressor of GA, suggest that GA biosynthesis and signalling is moderated during this interaction to balance growth and the defence response. Several recent studies have provided evidence of GA mediated plant immunity via an interaction with the JAZ proteins (Hong et al., 2012; Hou et al., 2010; Yang et al., 2012). In the presence of GA, DELLA the negative regulator of GA is rapidly degraded, resulting in the JAZ-MYC2 interaction. However, in the absence of GA, DELLA interacts with JAZ proteins, resulting in the disruption of the JAZ-MYC2 interaction also known as “release of repression” and activation of *MYC2*-mediated JA signalling (De Bruyne et al., 2014; Hong et al., 2012; Yang et al., 2012). The observed induction of DELLA and down regulation of GA20 oxidase and GA3 oxidase genes involved in GA biosynthesis suggest the plant mediated balance between growth and the defence response. However, DELLA mediated induction of *MYC2*-mediated defence against insect attack, contradicts the predicted defence response required for basal resistance against necrotrophic pathogen. However, further functional studies are required to understand the interaction of DELLA and JAZ proteins and GA mediated defence response in potato tuber.

Genes encoding the snakin proteins *Sn-1* and *Sn-2* were amongst the most significantly induced in response to *Pbr* ICMP19477 and were induced to a lesser extent in response to *Pba* SCRI1043. Members of the snakin protein family have been identified in a wide range of plants including the *Solanaceous* species tomato (Shi et al., 1992) and pepper (Mao, Zheng, et al., 2011). Studies to understand the role of snakin genes in the defence response have reported that the over-expression of *Sn-1* resulted in enhanced resistance of transgenic tubers to *Pcc* and *Rhizoctonia solani* (Almasia et al., 2008). Furthermore, treatment of transgenic GNL1-sensitive potato tubers with *D. chrysanthemi* resulted in enhanced resistance to this pathogen (López-Solanilla et al., 1998). The *snakin* genes have also been observed to play a significant role in cell division and maintaining the redox homeostasis during biotic stress. This was observed in *Arabidopsis* where *Sn-1* silencing resulted in altered redox balance and cell division (Nahirñak et al., 2012). Studies to characterise *Sn-1* and *Sn-2* in potato against various biotic and abiotic stress in different plant tissues have confirmed that *Sn-2* gene expression was higher than *Sn-1* (Meiyalaghan et al., 2014). This is consistent with the observed expression levels in this study. Given the known antimicrobial activity of these genes, and their induced expression in response to pectobacteria (significant induction in response to *Pbr* ICMP19477 and non-significant induction in response to *Pba* SCRI1043), it is speculated that these genes contribute substantially to the induced innate immune response in potato tubers against this pathogen and maintaining the redox balance within the cell.

The genes annotated as encoding *MYB* and *WRKY* TFs were DE in response to infection by pectobacteria. For instance, the gene predicted to encode *WRKY40* was significantly induced during infection by *Pectobacterium* infection (and wounding). In *Arabidopsis*, the closely related *WRKY* TFs *WRKY18*, 40 and 60 are involved in the negative regulation of ABA signalling (Pandey et al., 2010). Of particular note, *WRKY40* is involved in the regulation of *ABI4* and *ABI5*, genes involved in downstream ABA-related signalling (Liu, Yan, et al., 2012). Furthermore, *WRKY40 has* been related to the oxidative stress tolerance in *Fortunella crassifolia,* and transgenic plants over-expressing *WRKY40* show induced expression of the peroxidase gene (Gong et al., 2014). Peroxidase are involved in oxidative burst during plant pathogen interaction (O'Brien et al., 2012; Wrzaczek et al., 2013). Consistent with the induced

expression of *WRKY40*, genes annotated as peroxidase were also observed to be significantly induced during these interactions. Suggesting that induction of *WRKY40* may be involved in the oxidative stress tolerance in potato during *Pectobacterium* interaction, further functional studies are required to confirm the role of *WRKY40* during plant pathogen interaction in potato tubers. The gene predicted to encode *WRKY33* was also induced in response to *Pbr* ICMP19477. *WRKY33* has been related to the JA induced resistance to *B. cinerea* infection *in Arabidopsis* (Birkenbihl et al., 2012; Lippok et al., 2007; Zheng et al., 2006). Furthermore, WRKY 33 is involved in ET- and JA-mediated cross talk and camalexin biosynthesis (Birkenbihl et al., 2012). Taken together, the observed induction of *WRKY33* only in response to the pathogen suggest the role of *WRKY33* in active defence against this pathogen. However further studies are required to confirm the *WRKY33*–mediated camalexin biosynthesis in potato tubers and their possible role in defence against pectobacteria

In addition to the genes annotated as encoding *WRKY* TFs, genes predicted to encode *MYB*-related TFs were also induced in response to pectobacteria and mock-inoculation. Several *MYB*-related TFs have been reported for their role in plant defence, however, the regulatory mechanism by which the *MYB* TFs mediate the response still remains largely unknown. Genes annotated as *MYB108* were significantly induced in response to *Pbr* ICMP19477 and *Pba* SCRI1043. In *Arabidopsis*, induction of *MYB108* (At BOS1) has been related to defence against the necrotrophic fungus *B. cinerea* (Mengiste et al., 2003). Furthermore, infection of *Arabidopsis* with *D. dadantii* resulted in the induced expression of the *MYB108*, however, the transgenic *bos1* mutants resulted in induced susceptibility to this pathogen. It was also observed that the expression of *MYB108* was induced by the production of the bacterial PelB/C pectinases (Kraepiel et al., 2011). Recent studies on the defence response deployed by the host cotton plant to *Verticillium dahlia* have also observed the induced expression of *MYB108*, which was observed to have a positive interaction with CaM proteins (Cheng et al., 2016). Together with this data, the induction of the *MYB108* gene in potato tubers is suggestive of a DAMP-mediated PTI response to pectobacteria.

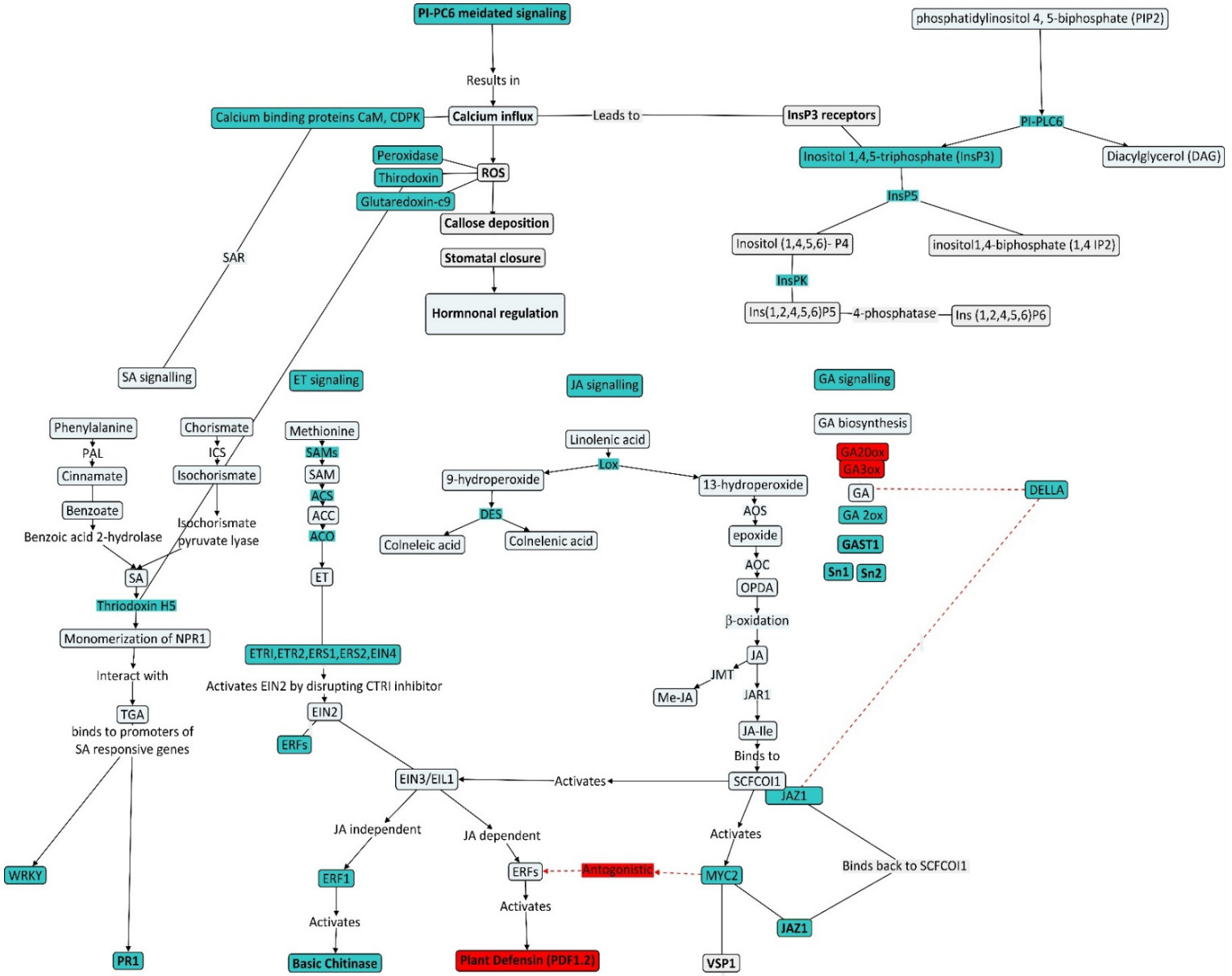


Figure 4.7: Diagrammatic representation of the early defence responses deployed by potato tubers upon infection with *Pectobacterium* spp., and their possible crosstalk.
The enzymes in the red box and blue box are genes down and up regulated respectively

Collectively, plant defence responses against biotic stress are tuned for defence against a particular stress. The host response observed in this transcriptome study provides an overview of DAMP-mediated PTI and the early defence responses deployed by the host to pectobacteria infection (Figure 4.7). The phytohormone-mediated defence response observed in this study also suggests a rather complex cross-talk employed by the plant in response to the pathogen. However, the JA-independent *MYC2* activated defence response is in agreement with the susceptible host pathogen interaction, where the host's defence response is diverted towards an active defence response against insect attack. Studies in *Arabidopsis* to understand COR-mediated host susceptibility confirmed that COR interacts with the COI1-JAZ complex and results in the activation of *MYC2*-mediated JA signalling (Katsir et al., 2008; Zheng et al., 2012). Given the mutual antagonistic cross-talk between the JA-SA pathways, in *P. syringae* COR manipulates the host defence response by activating the JA pathway and supressing the SA pathway, rendering the plant susceptible to the invading pathogen. The plant would normally depend on SA-mediated signalling for an active defence against biotrophic/hemibiotrophic pathogens like *P. syringae* (Glazebrook, 2005; Pieterse et al., 2012). *Pba* SCRI1043 and *Pbr* ICMP19477 carry *cfa* gene clusters (Bell et al., 2004; Panda et al., 2016). Given that CFA is a molecular mimic of MeJA, and is a component of COR (Bender et al., 1999), it is suggested that CFA, like COR, may play a central role in modulating the plant defence response. Indeed it would seem most likely that CFA influences the key defence JAZ-COI1 complex to manipulate production of defensin and other resistance proteins. Further studies are required to fully understand how CFA moderates host defences.

4.4.2.3 Metabolic profiling of potato tubers in response to *Pectobacterium* and exogenous CFA

Plant infection by necrotrophic pathogens elicits JA/ET related signalling, and these pathways mediate changes in secondary metabolism (Zhao et al., 2005). Secondary metabolites with antimicrobial properties are characterized as phytoalexins. Plants of a given species produce specific secondary metabolites, *Solanaceae* plants primarily produce sesquiterpene in response to abiotic and biotic stress (Dixon, 2001). Studies in tomato have identified the down regulation of mono- and sesquiterpene during mild and severe heat stress (Pazouki et al.,

2016). In contrast, potato tubers accumulate fungal toxic sesquiterpene in response to *P. infestans* (Bostock et al., 1981). These data are consistent with the results observed in this study, where the genes related to sesquiterpene synthase and germacrene C synthase (sesquiterpene) were induced in response to *Pectobacterium*, whereas the gene related to phenylpropanoid biosynthesis and flavonoid biosynthesis were significantly down regulated in response to the pathogen. The down regulation of the phenylpropanoid and flavonoid pathways was unexpected, as previous studies on the potato-*P. infestans* interaction have confirmed the induced expression and accumulation of the phenylpropanoid and flavonoid compounds in resistant genotypes (Yogendra & Kushalappa, 2016). Flavonoid biosynthesis results in the formation of a physical barrier, thus restricting pathogen penetration. Flavonoid production was also induced in *Malus* spp. in response to *E. amylovora* (Venisse et al., 2002) and in barley genotypes resistant to *F. graminearum* (Bollina et al., 2010; Kumaraswamy et al., 2012), whilst phenylpropanoid production in alfalfa resulted in resistance to *Colletotrichum trifolli* (Gallego-Giraldo et al., 2011). Given the rich history of phenylpropanoid and flavonoid production in resistance to pathogens, it would seem that either the susceptible nature of the interaction studied in this thesis results in these compounds not being produced (or even down regulated) or their production is inhibited in order to produce greater amounts of sesquiterpene, which may provide the greatest antimicrobial activity to pectobacteria. More comprehensive metabolic profiling and antimicrobial assays would hopefully confirm the role of sesquiterpene in the *Pectobacterium* mediated host defence response in potato tubers.

4.5 Conclusion

In this study, RNA-seq analysis using total RNA from potato tubers infected with *Pectobacterium* strains was sufficient to reveal the expected induction of DAMP-mediated PTI in response to these pathogens. Differential expression of various phytohormones including ET, SA and GA responsive genes was observed as well as the induction of genes related to the antimicrobial activity of the tuber, suggesting an active and complex defence response in the host, even in a susceptible interaction. Key details of this response were revealed, with a lack

of JA biosynthesis highlighted as well as the importance of the snakin genes (known to have activity against pectobacteria) in the innate response to these pathogens, and the possibility of ETI in response to at least one strain of *Pectobacterium* via a specific group of NBS-LRRs. Of greatest interest, however, was the seemingly central role of JA-independent *MYC2* activation in the interaction of potato tubers with the SRE. Given that COR has previously been shown to target the COI1 complex responsible for *MYC2* regulation, it would be interesting to test whether CFA also targets this complex to influence the outcome of the interaction of *Pectobacterium* with its plant host.

Chapter 5 The role of CFA in modulating the defence response in potato to *Pectobacterium atrosepticum* SCRI1043

5.1 Summary

In *P. syringae*, CFA is conjugated to CMA to form COR, a well characterised phytotoxin that mimics JA-Ile (Katsir et al., 2008). As a consequence, COR alters the defence response of the plant and facilitates pathogenesis. Various taxa of *Pectobacterium* also encode CFA including *Pba* SCRI1043 and *Pbr* ICMP19477. The CFA biosynthetic cluster is known to be a virulence determinant in these bacteria regardless of the absence of the CMA biosynthetic cluster, suggesting that CFA or a unique conjugate must confer virulence. Yet, no data exists on how CFA or a CFA conjugate might act as a virulence gene to subvert host defences. In this chapter, to begin to understand whether CFA functions in pectobacteria to manipulate host defences, a transcriptome analysis was performed to compare the defence response of potato tubers ('Summer Delight') infected with *Pba* SCRI1043 to tubers exposed to *Pba* SCRI1043ΔHAI2, a mutant in which HAI2 had been removed by CRISPR-Cas genome editing. HAI2 encodes the CFA biosynthetic cluster as well as the coronafacate ligase (CFL), which is predicted to conjugate CFA to an amino acid to form an active conjugate. Exogenous CFA (200 nM) was also applied to tubers for comparative purposes. Loss of HAI2 resulted in very few changes in differential expression in potato tubers, with the exception that *MYC2* was not DE when compared to the non-inoculated control. Interestingly, this gene was strongly up regulated in response to treatment with *Pbr* ICMP19477, but also with *Pba* SCRI1043. No differential expression of *MYC2* in plants exposed to *Pba* SCRI1043ΔHAI2 suggested that CFA or a CFA conjugate might target the *MYC2*-mediated JA response, which alters the production of key defence-related products used to combat bacterial infection. Transcriptome profiling also confirmed a significant overlap of CFA and *Pectobacterium*-regulated genes, with 40% of the *Pectobacterium*-regulated genes DE upon exposure to exogenous CFA. In particular, application of exogenous CFA resulted in differential expression of the JA- and ET-related

signalling pathways. In addition, *JAR1* was up regulated in response to exogenous CFA at 6 hpi. This result suggested that the presence of CFA induces its conjugation to an unknown amino acid. It is probable that the conjugate is necessary for downstream activation of JA signalling and in particular differential expression of *MYC2*. Consistent with this hypothesis, differential expression of *MYC2* upon exposure to CFA was not observed, probably because constitutive production of CFA (or the conjugate) is required to elicit the *MYC2*-related response. In *P. syringae*, COR is known to directly interact with the COI1-JAZ complex, resulting in the *MYC2* activation which determines JA-mediated downstream defence signalling.

5.2 Background

A variety of SRE including *Pba, Pcc, Pbr* and *Pw* (Duarte et al., 2004; Perombelon, 2002; Perombelon & Kelman, 1980; Pitman et al., 2008) cause soft rotting of potato tubers and potato blackleg disease. Although the PCWDEs are considered the major pathogenicity factors encoded by these pathogens, genome sequencing has identified further important virulence determinants including the *cfa* biosynthetic gene cluster (Bell et al., 2004; Glasner et al., 2008; Koskinen et al., 2012; Panda et al., 2015). The *cfa* biosynthetic gene cluster is present in the genomes of a number of SRE, which are all capable of eliciting blackleg (Panda et al., 2015; Panda et al., 2016). The presence of the *cfa* biosynthetic gene cluster in blackleg strains suggests that CFA supports colonisation of the stem (which has been confirmed by mutagenesis), but little is known about its mechanism of action or whether it also supports growth in potato tubers. This is particularly important, given SRE generally enter the plant through lenticels and wounds in tubers (Perombelon, 1992, 2002; Perombelon & Hyman, 1989; Perombelon & Kelman, 1980).

In *Pba* SCRI1043, CFA is harboured on HAI2 (Bell et al., 2004). Genetic characterisation of HAI2 showed that HAI2 is 97,875 bp in size, has a GC content of approximately 48% and encodes 99 predicted coding DNA sequences. The coding DNA sequences have strong similarities to those of known integrative and conjugative elements (Vanga et al., 2012), which are mobile

genetic elements that contribute to the evolution of pathogenic bacteria by mediating the horizontal gene transfer of virulence determinants. Studies to understand the mobilization of HAI2 have confirmed the induced excision and mobilization of the integrative and conjugative element during infection in potato stems and tubers (Vanga et al., 2015). Furthermore, deletion of HAI2 by CRISPR-Cas-based genome editing resulted in reduced virulence in stems (Panda et al., 2016), confirming the data of Bell et al., (2004), who showed CFA had a role in virulence using a *cfa6* and *cfa7* mutant. Interestingly, pathogenicity assays conducted in this study on potato tubers using the HAI2 mutant showed no significant reduction in disease incidence or disease symptoms (Chapter 3), indicative of a dispensable role for CFA during soft rot infection. Nevertheless, it was hypothesised that CFA might still interact with the host at the cellular level.

In *P. syringae*, the *cfa* and *cfl* gene cluster (also present in *Pba* SCRI1043) encodes enzymes responsible for the production of CFA and its ligation to CMA, which specifically results in COR (Bender et al., 1993). COR is produced as a part of the pathogen's phytotoxic complex, which includes many other phytotoxins including several CFA amide conjugates. COR forms an active part of the pathogen's arsenal of virulence factors and has been identified in several pathovars of *P. syringae* (Bender et al., 1999; Bender et al., 1993) suggestive of a broad host range for this phytotoxin. Consistent with this, the genes encoding CFA have been identified in other pathogens including *X. campestris* pv *phormiicola* (Mitchell, 1991) and *S. scabies* (Bignell et al., 2010). As previously stated, in *P. syringae*, COR is a structural and functional mimic of JA-Ile, the most active JA conjugate (Katsir et al., 2008). In addition to the proven ability of COR to mimic JA-Ile, the similarity of the CFA and the CMA moieties of COR to MeJA and 1-aminocyclopropane-1 carboxylic acid (ACC), respectively, has been confirmed (Brooks et al., 2004). Studies in tomato have reported that the activity of COR is dependent on the host's JA signalling pathway (Zhao et al., 2003). Indeed, studies in tomato to understand the activity of COR, CFA and MeJA have confirmed the COR-dependent activation of the ET-, JA- and auxin-related plant defence pathways, and a diverse range of biological functions including accumulation of anthocyanin, proteinase inhibitors, and production of secondary metabolites (Uppalapati et al., 2005; Uppalapati et al., 2007). COR also modulates chloroplast metabolism (Uppalapati et al., 2005).

Unlike *P. syringae*, pectobacteria lack the genes encoding CMA suggesting that COR is not produced by these pathogens. Yet, studies in *P. syringae* (Mitchell, 1984) and *S. scabies* (Fyans et al., 2015) have previously confirmed the secretion of both free CFA and CFA conjugates during *in vitro* fermentation while functional studies of the CFA biosynthetic cluster and HAI2 in *Pectobacterium* have shown that CFA or a CFA conjugate has a definite role in virulence even in the absence of CMA (Bell et al., 2004; Panda et al., 2016). Therefore, in this thesis, it was hypothesised that CFA plays a role in manipulation of the early phases of the host defence response in potato tubers, prior to the pathogen entering its necrotic phase (thereby enhancing plant susceptibility). To investigate this hypothesis, the transcriptional dynamics of potato tubers were studied after treatment with *Pba* SCRI1043ΔHAI2 and exogenous CFA (see Sections 2.2). Though CFA is not critical for the aggressiveness of pectobacteria on potato tubers, pectobacteria have been identified as seed borne pathogens. Thus, identification of the CFA-responsive genes in potato tubers offers the opportunity to study the toxin/effector-mediated defence response during the initial stages of the host-pathogen interaction.

5.3 Results

5.3.1 Transcriptional profiling of potato tubers in response to *Pba* SCRI1043ΔHAI2

Transcriptional profiling using DESeq2 (section 2.2.8.3.1) revealed a total of 558 DEGs in potato tubers ('Summer Delight') infected with *Pba* SCRI1043ΔHAI2 when compared to non-inoculation control tubers, across one or more sampling times. Comparison of these DEGs with those from tubers exposed to *Pba* SCRI1043 or the mock-inoculated control showed that a total of 163 genes were common across all treatments while 188, 164 and 45 genes were DE only in response to *Pba* SCRI1043, *Pba* SCRI1043ΔHAI2 or the mock-inoculated control, respectively (Figure 5.5). In addition, 191 genes were identified to be DE in response to both *Pba* SCRI1043 and *Pba* SCRI1043ΔHAI2.

Two separate approaches were used for further comparison of differential expression in tubers inoculated with *Pba* SCRI1043 or the HAI2 mutant. The first approach involved the use of hierarchical clustering, in which a total of 600 genes significantly DE in response to either *Pba* SCRI1043 or *Pba* SCRI1043ΔHAI2 were subjected to average linkage hierarchical clustering analysis using the default options present in Cluster 3.0. Clustering analysis allowed genes to be clustered based on the similarities in their expression patterns, yet no cluster specific to HAI2-mediated differential expression was observed (Figure 5.6). As an alternative approach, genes DE in response to the loss of HAI2 were identified by normalising the expression profile observed in potato tubers in response to *Pba* SCRI1043ΔHAI2 with the profile in tubers inoculated with *Pba* SCRI1043 across all time points. As a result of this analysis, a total of 30 genes were DE in tubers treated with *Pba* SCRI1043ΔHAI2 and *Pba* SCRI1043. No overlap of differential expression was observed between the time points. The transcription profile of DEGs are shown in Table 5.2. The relatively few observed DEGs between wild-type and the HAI2 mutant suggested that the loss of the island encoding the CFA did not impact extensively on the host-deployed defence response.

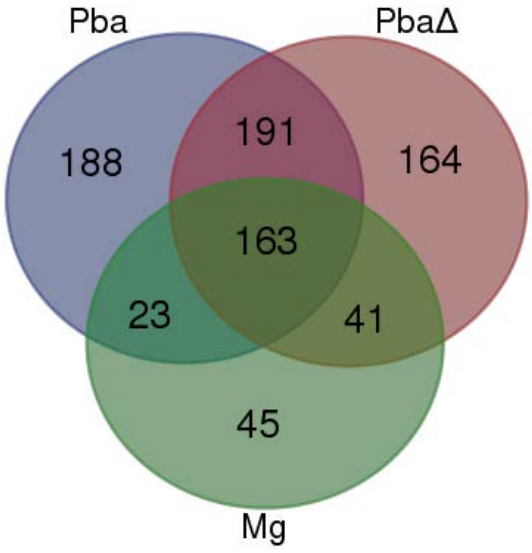


Figure 5.1: A Venn diagram showing the number of DEGs in potato tubers ('Summer Delight') in response to *Pba* SCRI1043, *Pba* SCRI1043ΔHAI2 and/or the mock-inoculated control (MG) across all time points.

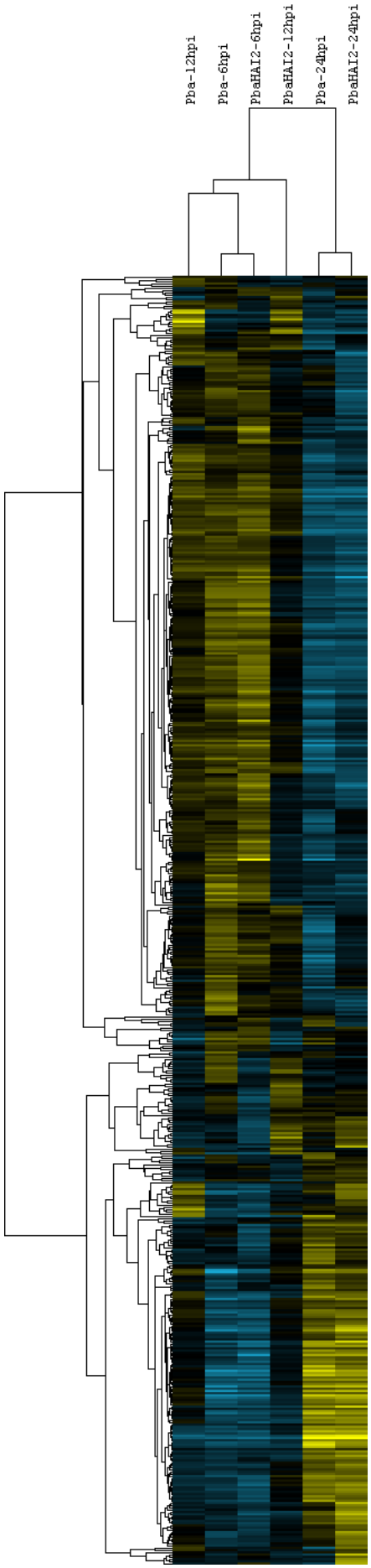


Figure 5.2: Hierarchical clustering of selected genes DEGs between *Pba* SCRI1043 and *Pba* SCRI1043ΔHAI2 at 6, 12 and 24 hpi.
Log2 fold changes observed using DESeq2 was used to cluster the DEGs using Cluster 3.0 software.

5.3.1.1 Annotation of differentially expressed genes in potato tubers in response to *Pba* SCRI1043ΔHAI2

The DEGs observed in response to *Pba* SCRI1043ΔHAI2 when normalised with *Pba* SCRI1043 were further profiled to identify the pathways associated with HAI2-mediated moderation of host defences. Of the DEGs, 50 % were identified as genes of unknown function (Table 5.1). Additional genes related to ET and JA signalling, secondary metabolite synthesis and 'resistance proteins' were DE in response to *Pba* SCRI1043ΔHAI2. Of most important note, genes annotated as *MYC2* were significantly down regulated in tubers exposed to *Pba* SCRI1043ΔHAI2 at 24 hpi when compared to tubers inoculated with the wild-type. In fact, no induction of *MYC2* was observed in response to the mutant when compared to the non-inoculated control whereas the wild-type elicited a substantial up regulation (log2 fold change = 2.5 at 24 hpi) of *MYC2*. *MYC2* was also induced by *Pbr* ICMP19477, which also carries the CFA biosynthetic cluster (Log2 fold change = 5 at 24hpi; see section 4.3.3.2, Table 4.3 for results). *MYC2* is a key regulator of the JA-dependent defence response against insect and herbivore attack (Dombrecht & Kazan, 2007). Down-regulation of this gene in the HAI2 mutant when compared to the wild-type strains suggested that HAI2 (and probably CFA) causes activation of *MYC2*. Of further note, ethylene responsive TF (Pti5) was significantly down regulated in response to *Pba* SCRI1043ΔHAI2. In potato, induction of ethylene responsive TF (Pti5) has been related to the defence response against aphids (Wu et al., 2015).

Table 5.1: A complete list of DEGs observed in response to *Pba* SCRI1043ΔHAI2 across all sampling time.
Note:- The *Pba* SCRI1043 was used to normalize DEGs. Numerical values represent the log2 fold change, positive values indicate the induced expression of the transcript when compared to the *Pba* SCRI0143 and negative values indicate the repression of the transcript when compared to the *Pba* SCRI0143.

Gene ID	Gene name	6 hpi	12 hpi	24 hpi
PGSC0003DMG400014880	ABC transporter family protein		-2.1	
PGSC0003DMG400028789	Conserved gene of unknown function	-2.4		
PGSC0003DMG400010622	Conserved gene of unknown function	-2.2		
PGSC0003DMG401020957	Conserved gene of unknown function			-2.2
PGSC0003DMG400008782	Conserved gene of unknown function			-2.1
PGSC0003DMG400025637	Conserved gene of unknown function			-2.1
PGSC0003DMG400023543	Conserved gene of unknown function			2.0
PGSC0003DMG400002791	Conserved gene of unknown function			2.1
PGSC0003DMG400003308	Cytochrome P450	-2.2		
PGSC0003DMG400018637	Erwinia induced protein 1	-2.2		
PGSC0003DMG400017233	ERF transcription factor(Pti5)		-2.0	
PGSC0003DMG400035878	Fatty acid desaturase			2.8
PGSC0003DMG400014879	Gene of unknown function		-2.1	
PGSC0003DMG400006804	Gene of unknown function			-2.9
PGSC0003DMG401018402	Gene of unknown function			-2.3
PGSC0003DMG400030582	Gene of unknown function			-2.3
PGSC0003DMG400009565	Gene of unknown function			-2.3
PGSC0003DMG400045863	Gene of unknown function			-2.1
PGSC0003DMG400018983	Gene of unknown function			-2.1
PGSC0003DMG400027636	Glutathione s-transferase		2.2	
PGSC0003DMG400017918	Glycine-rich protein			2.6
PGSC0003DMG401003767	Lysine-specific histone demethylase			-2.0
PGSC0003DMG400012237	MYC2			-2.6
PGSC0003DMG400006179	Nodulin family protein	-2.3		
PGSC0003DMG400011953	Non-specific lipid-transfer protein 2			2.1
PGSC0003DMG400015911	RNase H family protein			-2.1
PGSC0003DMG400015561	UPA23	-2.4		
PGSC0003DMG400018879	Wax synthase			2.2
PGSC0003DMG401019948	White-brown-complex ABC transporter family			-2.2

5.3.2 Transcriptional profiling of potato tubers in response to exogenous CFA

Gene expression profiles in potato tubers in response to exogenous CFA were compared to those in tubers infected with either *Pba* SCRI1043 or *Pbr* ICMP19477, two wild-type strains containing homologous CFA biosynthetic clusters. A total of 163 genes were DE upon exposure to both CFA and pectobacteria, whilst a further 170 genes were DE in response to CFA and *Pbr* ICMP19477, across one or more sampling times (Figure 5.1). These results indicated a greater degree of overlap between the CFA and *Pbr* ICMP19477. In addition, 51 genes were identified to be DE only in response to CFA. Besides these, 140 genes were DE response to wounding across all treatment (Figure 5.3).

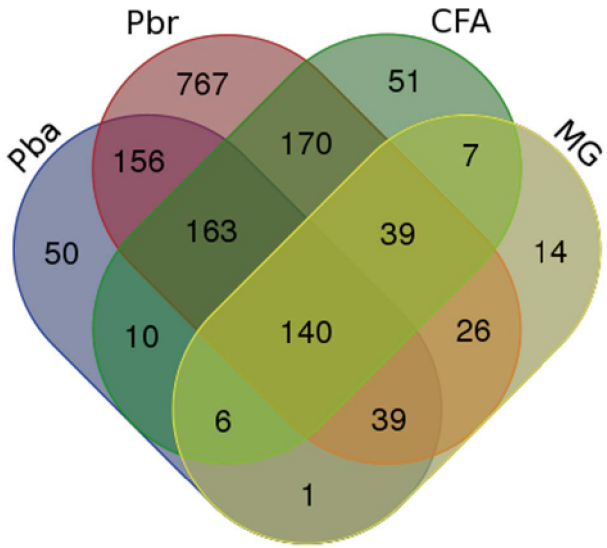


Figure 5.3: A Venn diagram showing a numerical representation of DEGs in potato tubers ('Summer Delight') in response to *Pba* SCRI1043, *Pbr* ICMP19477, exogenous Coronafacic acid (200 nM) and mock-inoculation, across all sampling time.

5.3.2.1 Annotation of the differentially expressed genes in potato tubers in response to exogenous CFA

The DEGs were divided into gene categories to establish whether specific functional categories were over-represented in response to each treatment. Gene Ontology (GO) implemented in BLAST2GO (3.3.5) was used for this purpose. Using this tool, approximately 60% of the DEGs identified in response to CFA were involved in 'biological processes'. Of these, genes relating to oxidation-reduction process, single-organism biosynthetic process, and regulation of transcription were over-represented across all three sampling times (Figure 5.4). Genes relating to photosynthesis were over-represented at 12 hpi, whilst genes associated with the 'defence response', including response to stress and response to abiotic stimulus, were DE at 24 hpi (Figure 5.4). These results suggested a biological activity of exogenous CFA on potato tubers. Furthermore, the activation of defence related genes suggested the toxin elicited a host defence response.

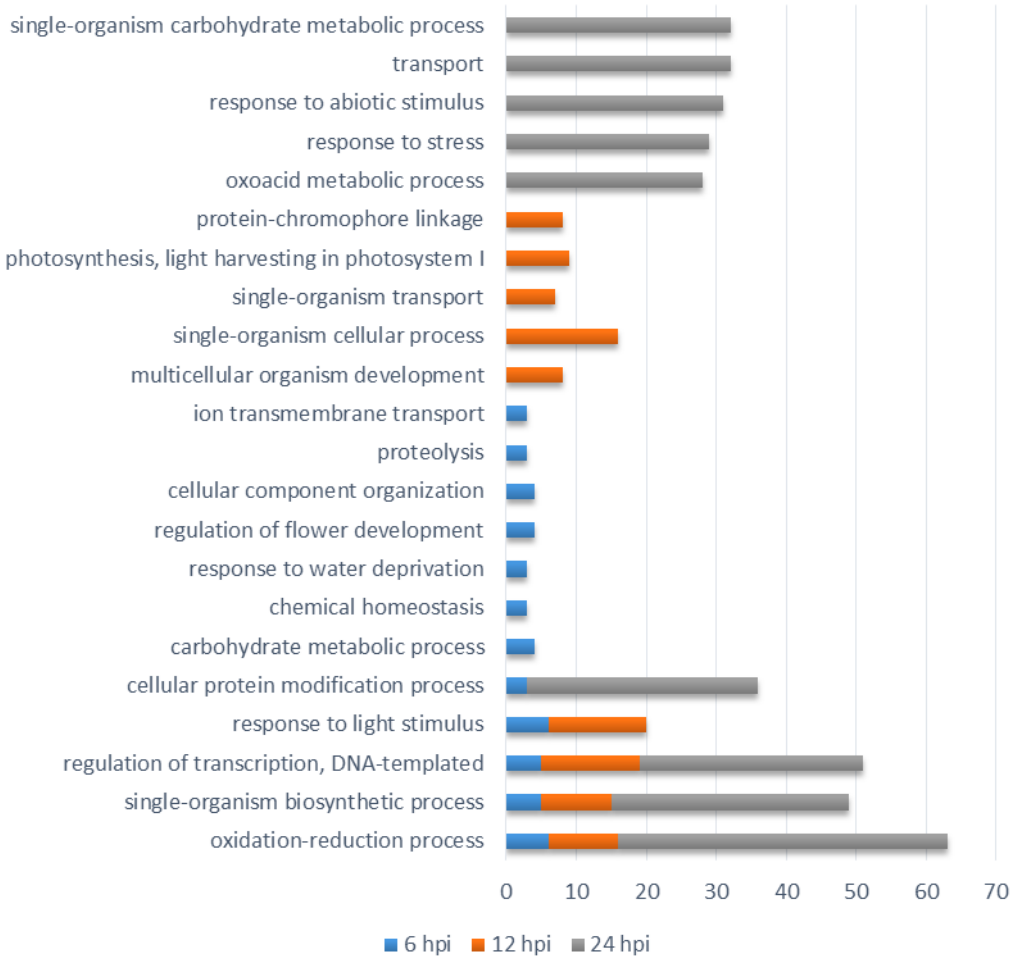


Figure 5.4: Gene ontologies for all DEGs categorised as involved in 'biological processes' in potato tubers ('Summer Delight') in response to exogenous Coronafacic acid (200 nM), across all sampling time.
BLAST, InterPro scan, and GO annotation implemented in BLAST2GO (3.3.5) were used to construct functional categories for the DEGs. The X axis represents the number of genes present in each category.

5.3.2.1.1 The early response in potato tuber in response to exogenous CFA

Consistent with the transcriptional dynamics observed in potato tubers in response to *Pba* SCRI1043 and *Pbr* ICMP19477, genes related to the PI-PLC signalling pathway were significantly induced in response to exogenous CFA (Table 5.2). For example, genes annotated as Inositol polyphosphate multikinase (PGSC0003DMG400014228), Inositol-1,4,5-triphosphate-5-phosphatase (PGSC0003DMG400024219), two key genes involved in the conversion of InsP3 to Ins(1,2,4,5,6)P5 were significantly induced in response to exogenous

CFA (pectobacteria and mock-inoculation). The induction of genes in the PI-PLC signalling pathway is consistent with the elicitation of a defence response to DAMPs/PAMPs perception and mechanical damage. However, the magnitude of fold change observed in response to pectobacteria and exogenous CFA were consistently higher than mock-inoculation.

In addition to the PI-PLC pathway, genes related to oxidative burst were significantly induced in response to exogenous CFA (and pectobacteria). In particular, genes annotated as ferredoxin-3 and chloroplast (PGSC0003DMG400023985) and glutathione-s-transferase/peroxide (PGSC0003DMG400031093) were significantly induced in response to CFA at 24 hpi. A non-significant induction of these genes was also observed in response to mock-inoculation, although the magnitude of the fold-changes observed in response to CFA (and pectobacteria) were consistently higher than mock-inoculation.

5.3.2.1.2 Hormonal modulation

The ethylene induced defence response

Studies in tomato have observed COR-mediated induction of ET biosynthesis and ET responsive signal transduction (Uppalapati et al., 2005). Consistent with these findings, genes related to ET signalling were induced in response to exogenous CFA (and to pectobacteria) (Table 5.1). For example, genes annotated as TSRF1 (PGSC0003DMG400017231, PGSC0003DMG400013402) (homologous to *ERF1*) were significantly induced in response to exogenous CFA (and pectobacteria) treatment. TSRF1 was also induced in response to mock-inoculation. This was not surprising, as previous studies have confirmed JA-independent activation of *ERF1* during both pathogen attack and mechanical wounding (Donnell et al., 1996; Lorenzo, Piqueras, Sánchez-Serrano, et al., 2003). In addition to *ERF1*, genes annotated as *ERF4* (PGSC0003DMG400010724, PGSC0003DMG400026821) and *ERF3* (PGSC0003DMG400022823), were up-regulated in response to CFA (and pectobacteria) at 24 hpi (Table 5.2). In *Arabidopsis*, *ERF4* and *ERF3* have been identified as the negative regulators of *PDF1.2*, a key defence related gene required for active defence against necrotrophic pathogens. The induction of these ERFs in response to pectobacteria and exogenous CFA,

suggest CFA-mediated induction of these *ERFs*. However, the role of these *ERFs* and their transcriptional control in potato remains to be studied.

The jasmonic acid induced defence response

COR targets the JA-mediated defence response (Uppalapati et al., 2005; Uppalapati et al., 2007; Uppalapati et al., 2008). In this study, divinyl ether synthase (*des*) (PGSC0003DMG400025158) gene involved in the linolenic acid metabolism, was up regulated in response to CFA at 24 hpi (Table 5.2). Interestingly, CFA also induced the expression of *JAR1* (PGSC0003DMG400019881) at 6 hpi (Table 5.2), but the genes related to downstream activation of the JA signalling pathway (including the *JAZ* and *MYC2* genes) were not DE. *JAR1* catalyses the formation of the biologically active JA-Ile (Staswick & Tiryaki, 2004). Thus, overall, the data on JA-related genes suggests a lack of JA biosynthesis in tubers which was also observed in response to pectobacteria. However, transient application of CFA does appear to induce expression of *JAR1* to form a conjugate.

The salicylic acid induced defence response

Previous studies on CFA- and COR-induced transcriptional responses suggested that COR and CFA mediates the down-regulation of the SA-related signalling pathway (Uppalapati et al., 2007; Zheng et al., 2012). In this study, no significant down-regulation of the SA signalling pathway was observed. Instead, several genes predicted to be involved in this pathway were up regulated (Table 5.2). For example, the genes annotated as encoding thioredoxin II (*TRXH5*) (PGSC0003DMG400019506) and Glutaredoxin-C9 (GRXC9) (PGSC0003DMG400009399), two key modulation genes involved in the monomerization of NPR1 and activation of TGA, respectively (Ndamukong et al., 2007; Tada et al., 2008), were induced in response to CFA. One of the three genes predicted to encode *PR1* (PGSC0003DMG400005109) were also up-regulated in response to CFA. Similar results were observed in response to *Pba* SCRI1043 and *Pbr* ICMP19477, whilst non-significant induction (Fold change < 2 log2 fold change) of these genes was also observed in response to mock-inoculation. These data suggest that the SA signalling pathway is elicited in response to

wounding, however, the magnitude of downstream signalling increases after treatment with exogenous CFA and pathogen attack.

5.3.2.1.3 Transcription factors

In addition to modulation of several hormone pathways, exogenous CFA resulted in differential expression of a variety of TFs (Table 5.2). For example, a gene predicted to encode the *MYB* TF was significantly DE in response to CFA. Induced expression of genes annotated as R2R3 *MYB108* (PGSC0003DMG400027157) was also observed in response to CFA, whilst genes annotated as encoding other *MYB* TFs, including the *MYB*-like DNA binding protein (PGSC0003DMG400025632) and *MYB114* (PGSC0003DMG400011294) were significantly down regulated in CFA-treated tubers. Finally, genes annotated as encoding *WRKY* TFs, including JA-induced *WRKY* (PGSC0003DMG400019824), were significantly induced in response to CFA. Many of these genes were DE in response to pectobacteria as well (Section 4.3.3.3) and showed non-significant induction (Fold change < 2 log2 fold) in response to mock-inoculation. These data might suggest that differential expression of these TFs occurs in potato in response to both pathogen attack and wounding, and perhaps extends to application of exogenous CFA.

Gene ID	Transcript Name	Mock-inoculated			*Pba* SCRI1403			*Pbr* ICMP19477			CFA (200 nM)		
		6 hpi	12 hpi	24 hpi	6 hpi	12 hpi	24 hpi	6 hpi	12 hpi	24 hpi	6 hpi	12 hpi	24 hpi
Phosphoinositol-phospholipase signalling pathway													
PGSC0003DMG400030360	PI-phospholipase C PLC6			1.6			**3.4**			**3.2**			
PGSC0003DMG400014228	Inositol polyphosphate multikinase		1.2			**2.3**			**2.2**	**2.6**			**2.5**
PGSC0003DMG400024219	Inositol-1,4,5-triphosphate-5-phosphatase			1.7			**2.4**			**2.2**			**2.1**
Oxidative burst													
PGSC0003DMG400031093	Glutathione S-transferase/peroxidase			1.8						**2.3**			**2.2**
PGSC0003DMG400023985	Ferredoxin-3, chloroplast			1.9			**2.2**			**2.7**			**2.5**
PGSC0003DMG400011098	Oxidoreductase	1.2			**2.2**			**4.4**			**3.5**		
Jasmonic acid biosynthesis and signalling													
PGSC0003DMG400010859	Lipoxygenase						1.5			1.3			
PGSC0003DMG400025158	Divinyl ether synthase			1.5			**2.1**			**3.6**			**2.1**
PGSC0003DMG400019881	JAR1										**2**		
PGSC0003DMG400015129	Defensin protein						**-2**			-1.9			-1.1
Salicylic acid signalling													
PGSC0003DMG400019506	Thioredoxin II	**2.4**	**2.6**	1.5	**2.7**	**2.7**	**2.5**	**3.0**	**2.9**	**2.4**		**2.2**	
PGSC0003DMG400009399	Glutaredoxin-C9	1.6	1.4	1.2			1.8		**2.2**	**3.7**			1.8
PGSC0003DMG400005109	PR-1						1.5			**2.4**			1.6
PGSC0003DMG400005110	PR1 protein						1.6			**2.8**			
PGSC0003DMG400005111	PR1 protein			1.1						**2.7**			
Ethylene biosynthesis and signalling													
PGSC0003DMG400000193	ACC synthase 2						**2.1**		2.7	4.1			2.4
PGSC0003DMG400017186	Ethylene receptor (EIN4)			1.1			1.9			2.1			1.8
PGSC0003DMG400010724	ERF transcription factor 4 B3		1.3	1.3			1.4			2.2			2.2
PGSC0003DMG400022823	Ethylene response factor 3			1.6			**2.1**			2.9			2.1
PGSC0003DMG400026821	Ethylene responsive factor 4									2.4			1.3
PGSC0003DMG400014594	Ethylene response factor B3			1.8			**2.3**			3.3			2.2
PGSC0003DMG400010285	Ethylene response factor 10 B3			2		**2.2**	**2.4**			3.4			2.2
PGSC0003DMG400017233	ERF transcription factor (Pti5)					**3.2**			2.1	3.4			1.1
PGSC0003DMG400029713	Transcription factor AP2-EREBP		1.1			**2.2**			2.2	2.2	1.4		
PGSC0003DMG400017231	Transcription factor TSRF1 (ERF1)			1.4			**3.5**			3.2			3.4
PGSC0003DMG400013402	Transcription factor TSRF1 (ERF1)			**2.3**			**3.0**			4.2			3.6
PGSC0003DMG400025282	AP2/ERF transcription factor	1.4	1.6		**2.3**			3.5			3		
PGSC0003DMG400026261	ATERF-2/ATERF2/ERF2			1.6		**2.3**				3.5			2.5
PGSC0003DMG400026046	ERF transcription factor 13 (B3)												2.3
PGSC0003DMG400002272	Transcription factor AP2-EREBP (B3)		1.1			**3.2**	**2.1**		2.2	3.2			2.8
Transcription Factors													
PGSC0003DMG400019824	JA-induced WRKY protein	1.7	1.6	1.7		**2.3**	**3.7**		**3.2**	**4.1**		**2.1**	**2.7**
PGSC0003DMG400031140	WRKY transcription factor		1.1									**2**	
PGSC0003DMG400013966	MYB transcription factor	-1.3		-1.1				**-2.4**		**-2.5**			**-2.1**
PGSC0003DMG402004611	MYB transcription factor (MYB 108)			1.2			**2.7**			**2.6**			**2**
PGSC0003DMG400011294	MYB transcription factor MYB114	-1.7	-1.8	-1.9		**-4.7**	**-2.3**		**-4.2**	**-4.3**		**-3.8**	**-2.9**
PGSC0003DMG400025632	Myb-like DNA-binding protein	-1.6	-1.9	-1.4		**-2.6**			**-2.2**			**-2.1**	
PGSC0003DMG400027157	R2R3 transcription factor MYB108 1			1.3						**3.1**			**2.4**

Table 5.2: A list of selected DEGs in response to mock-inoculation, *Pba* SCRI1043, *Pbr* ICMP19477 and exogenous Coronafacic acid (200 nM) across all sampling time.

Note:-Non-inoculated control was used to normalize DEGs. When value is not present the data did not qualify the quality control.
Numerical values represent the log2 fold change, positive values indicate the induced expression of the transcript when compared to the Non-inoculated control.

5.3.2.1.4 Secondary Metabolite

Consistent with the transcriptional dynamics observed in potato tubers in response to *Pba* SCRI1043 and *Pbr* ICMP19477, genes related to the phenylpropanoid biosynthesis, flavonoid biosynthesis and terpenoid biosynthesis were significantly DE in response to exogenous CFA (Table 4.5). For example, genes annotated as Epidermal germacrene C synthase (PGSC0003DMG400006694), Cembratrienol synthase 2a (PGSC0003DMG400011777), two key genes involved in the germacrene (sesquiterpene) biosynthesis were significantly induced in response to exogenous CFA (pectobacteria and mock-inoculation). The induction of these genes is consistent with the previous studies where sesquiterpene have been identified as a major antimicrobial compound/ phytoalexins produced by *Solanaceous* plants and are induced during PTI, ETI and mechanical damage. However, the magnitude of fold change observed in response to pectobacteria and exogenous CFA were consistently higher than that observed in response to mock-inoculation.

5.4 Discussion

Genome sequencing of *Pba* SCRI1043 by Bell et al (2004) identified CFA biosynthetic cluster encoded on a HAI, which was subsequently shown to be a virulence factor for this pathogen. Recent studies by Panda et. al (2016) confirmed the ubiquitous nature of this HAI across various strains of *Pba*, suggesting that CFA along with the island probably provides a significant benefit for this pathogen, at least in some ecological niches. Consistent with this hypothesis, the deletion of HAI2 from the genome of *Pba* SCRI1043 resulted in a decrease in the incidence of disease symptoms on potato stems (Panda et al., 2016). The inactivation of *Topo IIIβ*, a gene on HAI2 encoding a homologue of topoisomerase III, also resulted in the induced excision of the island leading to reduced virulence of *Pba* on potato stems (Vanga et al., 2012). Nevertheless, a precise mechanism of action for HAI2 or CFA remained unclear. Given that *Pectobacterium* is a seed born pathogen, in this study the impact of HAI2 and CFA on potato tubers was investigated using transcriptomics.

Comparison of the transcriptional profiles in tubers treated with *Pba* SCRI1043ΔHAI2 and the non-inoculated controls identified 708 DEGs across all sampling points. Pathway analysis of the DEGs between the wild-type strain and the HAI2 mutant failed to identify significant differences in the global defence response to *Pba* SCRI1043ΔHAI2 pathogens. During the pathogenicity assays no significant differences in disease symptoms were observed in response to *Pba* SCRI1043 and *Pba* SCRI1043ΔHAI2. Given that induction of the necrotic lesions and subsequent disease development induces DAMP-mediated defence response in host, the lack of differences in the differential expression pattern observed in response to *Pba* SCRI1043ΔHAI2 was not completely surprising. Furthermore, having previously identified the insignificant role of CFA in the virulence of this pathogen in tubers, it was speculated that CFA would have a subtle impact on the tuber defence response. Thus, to identify the CFA-mediated impact on host defence, the transcription profile observed in response to *Pba* SCRI1043ΔHAI2 was normalised with that observed in response to *Pba* SCRI1043. Very few genes were identified to be DE during this comparison. Of particular note was the significant down regulation of *MYC2* in response to *Pba* SCRI1043ΔHAI2 (i.e. no differential expression of *MYC2* in *Pba* SCRI1043ΔHAI2 tuber when normalised with non-inoculation control).

MYC2 acts as the regulatory hub within the JA signalling pathways (Kazan & Manners, 2008, 2013; Staswick, 2008) Studies to identify the direct and indirect regulation of *MYC2* during JA signalling in *myc2* mutant have confirmed the differential expression of large sets of JA response genes (Kazan & Manners, 2013). Previous studies also suggest that *MYC2* acts as positive regulator of defence against herbivore, oxidative stress tolerance and a negative regulator of pathogen mediated defence response and JA mediated secondary metabolite biosynthesis (Dombrecht & Kazan, 2007). Studies to understand the phytohormone mediated defence response in various host have confirmed the mutually antagonistic interaction between *MYC2* and *ERF1* branch of JA signalling pathway suggesting that *MYC2* and *ERF1* represent two separate nodes within the JA signalling pathway that allow for the activation of the these pathways by various stimuli (Pieterse et al., 2012). Induction of the JA-mediated *ERF1* is responsible for the active defence against the necrotrophic bacteria and fungus. Lack of expression of *MYC2* in response to *Pba* SCRI1043ΔHAI2 (when compared to non-inoculated control SCRI1043), and induced expression during infection with the wild-type strains *Pba* SCRI1043 and *Pbr* ICMP19477 suggest HAI2-mediated activation of *MYC2*. Studies in

Arabidopsis, have confirmed the interaction of COR to COI1-JAZ complex and subsequent activation of the *MYC2* mediated JA signalling (Katsir et al., 2008; Melotto et al., 2008). Taking into consideration that CFA is the component of COR and is encoded in the HAI2 island, it is hypothesised that like COR, CFA may well interact with COI1-JAZ complex resulting in the induction *MYC2*, an inappropriate defence response to this necrotrophic pathogen.

In addition, ET response TF homologous to TF Pti5 in tomato was induced only in response to *Pectobacterium* and exogenous CFA (Table 5.2) but was significantly down regulated in HAI2 mutant (Table 5.1). Recent studies by Wu et.al (2015), suggest phytohormone independent and *Pti5*-mediated defence against potato aphids in tomato plants. Furthermore, the overexpression of *Pti5* in tomato plants accelerates the expression of *PR1* and *PR2* but not *PDF1.2* during infection with *P. syringae* (Gu et al., 2002; He et al., 2001). Taken together, the differential expression observed in this study suggests ET-independent and HAI2-mediated induction of Pti5 during potato-*Pectobacterium* interaction. We speculate that, if *MYC2* functions similarly in potato, CFA mediated activation of the *MYC2* and Pti5 may potentiate the plant defence to pest and insect attack there by exhausting the host resources and rendering the host susceptible to necrotrophic infection.

Further, the results observed in the *Pba* SCRI1043ΔHAI2-mediated differential expression was validated by comparing the transcriptional profiles between exogenous CFA and non-inoculated. This was done in an attempt to observe the impact of exogenous CFA on the host defence response and thus to identify their role during potato-*Pectobacterium* interaction. The biological activity and the dose response assay using exogenous CFA on potato tubers did not demonstrate a significant induction of disease symptoms in potato tubers. However, exogenous CFA-mediated expression profiling in potato tubers suggested that CFA regulated 40% of the pectobacteria induced genes, confirming the biological activity of CFA in potato tubers. In this study, genes related to ET signalling pathway and JA biosynthesis were observed to be DE in response to exogenous CFA.

Given that CFA is a molecular mimic of MeJA, and since previous studies have identified induced expression of JA pathway in response to COR (Uppalapati et al., 2005; Uppalapati et al., 2007), it was expected to identify a significant induction of JA biosynthetic and JA

mediated signalling pathway. However, genes related to the JA biosynthesis were not DE in this study. This was consistent with the activation of *lox 1; lox 1* in potato belongs to the *9-lox* and is not involved in the JA biosynthesis. Alternatively, *9-lox* along with *des* catalyse the conversion of linoleic and linolenic acid to colneleic acid and colnelenic acid, respectively (Vellosillo et al., 2007). The lack of differential expression of JA biosynthesis genes was not completely unexpected, given that the transcriptome profiling in response to *Pectobacterium* resulted in similar differential expression pattern. Transgenic tobacco plants, expressing antisense to *9-lox*, displayed increase susceptibility to infection by *P. parasitica* (Fammartino et al., 2007; Rancé et al., 1998). Further infiltration of potato leaves with *P. syringae* and *P. infestans* also resulted in the induced accumulation of *9-Lox* and *des* (Stumpe et al., 2006; Stumpe et al., 2001). In this study, the genes encoding *des* were induced in response to pectobacteria and exogenous CFA, and to a lesser extent in mock-inoculated control. Collectively, the lack of differential expression in JA biosynthetic genes, and the induction of *9-lox*, suggests the lack of bioactive JA, and involvement of *des* in wound and pathogen mediated defence in potato tuber.

Studies in tomato have identified CFA as biologically less active than COR and other CFA conjugates, concluding that conjugation of CFA to amino acid is essential for the full biological activity of this compound (Uppalapati et al., 2005). Recent studies in *S. scabies* have also confirmed that, when compared to CFA, CFA-isoleucine (CFA-Ile) purified from the culture supernatants were more biologically active (Fyans et al., 2015). Consistent with these data, we observed the induction of *JAR1* in response to exogenous CFA during the early time points. In, *Arabidopsis JAR1* has been identified as jasmonate amino acid synthase, involved in the conjugation of JA to Isoleucine resulting in JA-Ile, the bioactive conjugate of JA (Staswick et al., 2002). In addition the induced expression of *JAR1* in response to exogenous CFA during the early time points and non-significant downregulation during the later time points confirms CFA mediated induction of *JAR1*. These results also suggest that the conjugation of CFA to the amino acid is required for the biological activity of this compound.

Previous studies by Thines et.al (2007) have demonstrated that JA-Ile but not JA or MeJA is required for the activation of the COI1 complex and the JA-mediated activation of *MYC2*, suggestive of the importance of JA-Ile in activating the JA signalling pathway. Interestingly,

the gene related to the downstream induction of the JA signalling pathway including the *JAZ* and *MYC2* were not observed in response to CFA. It is likely that the rate of CFA-amino acid metabolism in potato tubers may directly influence the rate of JA responsive genes and suggest that coordinate production of CFA-amino acid is required for the effective activation of downstream response.

Furthermore, in this study induction of *JAZ* and *MYC2* but not *JAR1* was observed in response to *Pectobacterium* infections. Both *Pba* SCRI1043 and *Pbr* ICMP19477 carry the *cfl* gene responsible for the ligation of the CFA to the CMA; given that *Pectobacterium* lack the *cma* gene we hypothesise that *Pectobacterium* produces CFA-amino acid conjugates during soft rot infection, thus eliminating the need for JAR1-mediated amide conjugation. And hence it is suggested that the coordinate production of CFA-conjugate is required for the consequent activation of the *MYC2*-mediated defence response during wild-type infections.

In this study we observed the induction of *ERF1*, *ERF2*, *ERF3* and *ERF4* in potato tubers in response to exogenous CFA and *Pectobacterium*. A wide range of biological activity has been described for the ERF family proteins, *ERF1*, an instigator for ET and JA/ET response is the primary transcriptional activator in ET signalling pathway (Lorenzo, Piqueras, Sanchez-Serrano, et al., 2003). Overexpression of *ERF1* and ERF2 results in the induction of defence genes PDF1.2 and class I chitinase, consequently increased resistance to the necrotrophic pathogens (Berrocal-Lobo & Molina, 2004; McGrath et al., 2005). By contrast the induction of *ERF3* and *ERF4* results in the negative regulation of the JA/ET mediate defence response (McGrath et al., 2005; Ohme-Takagi et al., 2000). Studies in *Arabidopsis* have also identified *ERF4* as a negative regulator of the JA/ET-responsive defence genes and resistance to necrotrophic pathogen *Fusarium oxysporum* (Berrocal-Lobo & Molina, 2008; McGrath et al., 2005). The inactivation of the *MYC2* and *ERF4* has shown to induce resistance to necrotrophic pathogen *Fusarium oxysporum* (Anderson et al., 2004). Interestingly, treatment of *ERF4*-overexpression plant with MeJA resulted in the downregulation of *PDF1.2*, but failed to show any change in the class I chitinase expression pattern. Further, T-DNA insertion in the *ERF4* resulted in the 30 fold increase in *PDF1.2* expression (McGrath et al., 2005). ET responsive TFs, *ERF4* and *ERF3* are activated by *EIN2* and are independent of *EIN3* activation (Ohme-Takagi et al., 2000). In addition ERFs have also been proposed to act downstream of NPR1,

and result in the SA-mediated suppression of JA inducible PDF1.2 (McGrath et al., 2005). Collectively, the results from this study suggests existence of two separate branch for *ERF1*-mediated activation of chitinase and plant defensin (*PDF1.2)*. Activation of multiple genes encoding the *ERF1* and the difference in the magnitude of the induction in response to treatments suggest that *ERF1* gene respond differently to various stimuli including mechanical wounding, pathogen attack and abiotic stress. The results from this study indicates that the induction of *ERF1* and class 1 chitinase is wound responsive, however the higher log fold change observed in response to *Pectobacterium* and exogenous CFA also suggest the role of chitinase in pathogen-mediated defence response in tuber. Further the induction of the *ERF4* in response to *Pectobacterium* and exogenous CFA is consistent with down regulation of defensin (*PDF1.2)*. The results observed in this study suggest the role of CFA/CFA conjugate in manipulating the host defence response. However more comprehensive structural studies on the interaction of CFA with COI1-JAZ complex is required to ascertain the CFA-mediated activation of inappropriate defence response. .

5.5 Conclusion

In this study RNA-seq analysis using total RNA from potato tubers infected with *Pba* SCRI1043ΔHAI2 strain and exogenous CFA was sufficient to reveal the HAI2 and CFA-mediated defence response at 6, 12 and 24 hpi. The results from this study provided data to fuel our speculations on the mode of the action of CFA during Potato-*Pectobacterium* interaction. The expression profile of *MYC2*, observed in this pathosystem, suggest that CFA manipulate the host defence response by causing inappropriate defence response from the host. Further, the results also confirm the need of conjugation of CFA to the amino acid for increase bioactivity, hence further studies are required to confirm the production of CFA-conjugates *in planta*.

Chapter 6 *In planta* expression and detection of CFA and CFA conjugates

6.1 Summary

In chapter 6, the production of CFA by pectobacteria during blackleg and soft rot infection was studied by examining the *in planta* expression of the *cfa* biosynthetic cluster in *Pba* SCRI1043 using qRT-PCR, and the production of CFA by mass spectroscopy. Tissue-specific *in planta*-induced transcription of the *cfa* biosynthetic gene cluster was observed during soft rot and blackleg infection, although the expression of *cfa6* was significantly higher in the stem. The greater expression of *cfa* in the stem may explain why no reduction in virulence was observed in tubers when CFA production was inactivated in *Pba* SCRI1043 whereas deletion of the *cfa* genes resulted in lower virulence during blackleg infection. Consistent with the *in planta*-induced expression of the *cfa* gene cluster, GC-MS analysis of infected tissues confirmed the production of CFA in both tubers and stems. In addition to CFA, CFA-L-Valine (CFA-Val), a CFA-amino acid conjugate was also detected using GC-MS analysis during blackleg infection. The production of CFA conjugates (coronafacoyl amide) by *Pectobacterium* spp. *in planta* was consistent with the results observed in the transcriptomic study in chapter 5, which suggested that conjugation of CFA to amino acid is essential for the bioactivity of CFA in *Pectobacterium* spp. and for CFA-mediated manipulation of the downstream components of the host JA signalling pathway.

6.2 Background

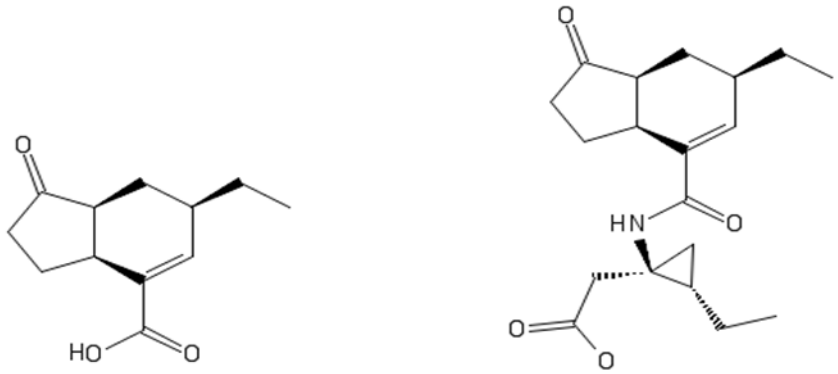
Phytopathogenic bacteria and fungi secrete various phytotoxic compounds that interfere with plant biochemical functions and produce symptoms like chlorosis or necrosis in a localised area of the plant. These symptoms were traditionally used for diagnosis of a particular disease

(Durbin, 1991), but the molecular impact of these toxins on host tissue often remained unknown. The two distinct SRE potato pathogens *Pba* SCRI1043 and *Pbr* ICMP19677 carry putative phytotoxin biosynthetic clusters with similarity to CFA from *P. syringae* (Bell et al., 2004; Panda et al., 2016) (see Section 5.2 for more details). Their role in virulence was confirmed when pathogenicity assays on potato stems using either *Pba* SCRI1043ΔHAI2 or strains carrying a mutation in *cfa6* and *cfa7* showed a significant decrease in disease symptoms (Bell et al., 2004; Panda et al., 2016). Furthermore, the presence of the CFA cluster in numerous other SRE isolated from potato plants with blackleg disease (Slawiak & Lojkowska, 2009), and the failure of *Pcc* strains that lack the CFA gene cluster to cause stem infection (Pitman et al., 2008), suggested that the CFA gene cluster was important for virulence of SRE on potato stems more generally. Little knowledge about the role of CFA in virulence of *Pectobacterium* spp. on potato tubers existed, however.

Chapter 3 of this thesis, demonstrated that in a soft rot assay on potato tubers, *Pba* SCRI1043ΔHAI2 did not display reduced virulence on the host. Also, application of exogenous CFA to potato tubers showed no necrosis at the site of inoculation, suggesting the role of CFA during soft rot disease progression was insignificant. In *P. syringae*, however, CFA is a component of COR, a phytotoxin that modifies hormonal defence pathways in the host (Bender et al., 1999). Consistent with this activity, a transcriptome analysis of potato tubers inoculated with *Pba* SCRI1043ΔHAI2 or with exogenous CFA (Chapter 5) showed modifications in expression of biotic stress-related genes, including those involved in JA and ET biosynthesis, and regulation of TFs. In particular, induction of *JAR1* (involved in the ligation of JA to amino acid) at early sampling times after exposure to CFA, suggested that the ligation of CFA to an amino acid might be required for the bioactivity of CFA. Furthermore, the down regulation of *MYC2* in *Pba* SCRI1043ΔHAI2 when compared to *Pba* SCRI1043 suggested that this conjugated form of CFA might mediate *MYC2* activation. Consistent with this hypothesis, studies in tomato using *P. syringae* have confirmed the COR-mediated degradation of the JAZ protein and *MYC2* activation (Zheng et al., 2012). The interaction of COI1 and COR was primarily due to COR acting as a structural mimic of JA-Ile (Geng et al., 2014; Katsir et al., 2008; Sheard et al., 2010; Uppalapati et al., 2007; Zheng et al., 2012).

Taking into consideration that CFA acts as a molecular mimic of MeJA (Brooks et al., 2004; Uppalapati et al., 2005), the differential expression observed in potato tubers upon exposure to exogenous CFA and the bacteria themselves, and the differences in the response of tubers and stems to infection with the *Pba* SCRI1043 and the HAI2 mutant, it was proposed that the ligation of CFA to an unknown amino acid is required for bioactivity of this phytotoxin and that the conjugated form induces the *MYC2*-mediated JA signalling pathway.

Pseudomonas spp. infect host plants and secrete various toxins including COR (Bender et al., 1999). Indeed, it was attempts to study the chlorosis-inducing phytotoxin in *P. syringae* pv *atropurpurea* that resulted in the *in vitro* purification and structural characterisation of COR (Figure 6.1), and an understanding of the bioactivity of purified coronafacoyl compounds using hypertrophy assays (expansion of cells) on potato tuber tissue (Ichihara et al., 1977). In addition to COR, coronafacoyl compounds including CFA were isolated from *P. syringae* pv *atropurpurea* (Ichihara et al., 1977). COR was subsequently purified from different pathovar (pv) of *P. syringae*; *P. syringae* pv *atropurpurea* (Italian ryegrass) (Ichihara et al., 1977), *P. syrinage* pv *glycinea* (soybean) (Mitchell & Young, 1978) and *P. syringae* pv *tomato* (tomato) (Mitchell et al., 1983). Norcoronatine, CFA-Val and CFA-Ile (Mitchell, 1984, 1985) were also purified from *in vitro* culture supernatants of *P. syrinage* pv *glycinea* and *P. syringae* pv *atropurpurea*. Studies to characterise the conjugation of CFA to these amino acids hypothesised that, under *in vitro* culture conditions, CFL had high specificity to CFA when compared to the amino acids utilised in the ligation process. This was confirmed when the addition of amino acids to the *in vitro* liquid cultures resulted in the increase in detectable quantities of the corresponding CFA conjugates and reduction in the quantities of CFA, COR and CFA-Val (Mitchell & Frey, 1986). The structure of COR, CFA and CFA-Val, and the characteristic peak of their methylated esters observed in *P. syringae* are represented in Figure 6.1 and Table 6.1, respectively.



Coronafacic acid (CFA)

Coronatine(COR)

Figure 6.1: Structures of coronafacic acid (CFA) and Coronatine (COR)

Table 6.1: Mass spectroscopy peaks and molecular ions of coronafacoyl compounds; methyl ester derivatives from *P. syringae* culture supernatants (Bender et al., 1989).

Compound	Molecular ion (*m/z*)	Diagnostic fragment ions
COR	333	301, **191***, 163
CFA	**222***	193, 191, 190, 163
CFA-Val	321	301, **191***, 163

*- Most abundant ion.

CFA and other *N*-coronafacoyl-L-amides were also identified in other bacteria including *X. campestris* pv *phormiicola* (New Zealand flax) (Mitchell, 1991) and *Streptomyces scabies* (potato) (Fyans et al., 2015). For example, CFA-Val and CFA-Ile were purified from *in vitro* cultures of *X. campestris* pv *phormiicola* (Mitchell, 1991). Chromatography studies in *S. scabies* also reported the production of CFA and CFA-Ile (Fyans et al., 2015). Culture extracts from a *S. scabies* strain overproducing CFA-Ile, were shown to induce hypertrophy in potato tuber tissue and stunted growth in radish seedlings, in a manner similar to purified COR from *P. syringae*, whereas extracts from strains lacking the *cfl* gene failed to show any bioactivity (Bender et al., 1989; Fyans et al., 2015). Taken together, the data from studies on *P. syringae* and *S. scabies* suggested that coupling of CFA to an amino acid was essential for bioactivity in plants (Fyans et al., 2015). Though the production of CFA conjugates in *P. syringae* and *S.*

scabies have been reported in media supplemented with amino acid, the failure to produce these conjugates in MM media indicated that the amino acids were not metabolically available for conjugation (Fyans et al., 2015; Mitchell & Frey, 1986). Furthermore, it was hypothesised that invasion of the host plant by these organisms during disease development may provide the amino acids required for the coupling reaction and thereby increase the biosynthesis of the coronafacoyl compounds either in addition or in the place of COR. Unfortunately, *in planta* production of the specific *N*-coronafacoyl-L during infection by *P. syringae* and *S. scabies* has not been confirmed, and this hypothesis remains to be proven.

6.3 Results

6.1.1 Tissue-specific induction of *cfa* gene in potato

Previous studies in *Pba* SCRI1043 confirmed the *in planta* (stem) induction of *cfa6* and *cfa7,* consistent with their role in virulence of the pathogen during blackleg infection (Vanga et al., 2012). The influence of *cfa* as a virulence factor during soft rot infection was not studied, however. Thus, to understand the role of *cfa* upon infection of the plant through tubers, the expression of the *cfa* biosynthetic cluster *in vitro* and *in planta* (tuber and stem) were compared. The *cfa* gene expression was measured using qRT-PCR.

Initially, the qRT-PCR protocols for the detection of *cfa6* and *cfa7* were optimised. The amplification efficiencies and regression values for each gene and the internal control *ffh* in these experiments are listed in Table 6.2. Expression of *cfa6* and *cfa7* were highly correlated (r = 0.99) in various conditions, suggesting either of these genes could be used to study the transcription of the *cfa* biosynthetic gene cluster in more detail. Thus, qRT-PCR of *cfa6* was used in subsequent experiments to estimate the expression the *cfa* biosynthetic cluster.

Table 6.2: Amplification efficiency and regression value (R^2) for each target gene used in qRT-PCR studies, as observed in MM media.

Gene	Efficiency	R^2
ffh	1.725	98.85
cfa6	1.715	98.47
cfa7	1.7	98.22

To compare the *in vitro* and *in planta* expression of *cfa*, *Pba* SCRI1043 was either inoculated into potato tubers or stems ('Summer Delight'), or cultured in MM media (section 2.4.1). The regression (R^2) values for both genes (*cfa6* and *ffh*) were above 99%, and amplification efficiencies were consistent (> 1.85) (data not shown). There were significant differences in the transcription of *cfa6* between the treatments ($p<0.001$ for an overall test). The normalised $\log_{10}$ quantities of the *cfa6* gene in *in planta* (stem) treatments were significantly ($p < 0.001$) greater than those observed *in vitro*. Though the normalised $\log_{10}$ quantities of the *cfa6* gene *in planta* (tubers) was 4X greater than those *in vitro,* these differences were not statistically significant ($p = 0.056$). The results of analysis of variance of the normalised $\log_{10}$ quantities are shown in Table 6.3.

Table 6.3: Back-transformed means (95% confidence limits) for the normalised log10 quantities of the cfa6 gene in planta and in vitro.

Treatment	Mean of three replicates
in vitro	0.06 (0.02, 0.17)
in planta (tuber)	0.25 (0.08, 0.73)
in planta (stem)	7.19 (2.43, 21.28)

6.1.2 Detection of CFA *in planta* during infections with *Pectobacterium* spp. using GC-MS

To confirm if the expression of the *cfa* biosynthetic gene cluster related to the production of the CFA metabolite, the *in planta* production of CFA and CFA conjugates were studied by GC-MS of the methylated metabolites obtained from freeze dried infected plant material (section 2.4.5), developed for *in planta* detection of phytohormones (Schmelz et al., 2004).

In an initial GC-MS experiment for CFA detection, rotten tuber tissues ('Ilam Hardy') infected with *Pbr* ICMP19477 and stem tissues ('Ilam Hardy') infected with *Pba* SCRI1043 were used as samples. The CFA standard (kindly provided by Robin Mitchel and Dave Greenwood, Plant & Food Research), along with the metabolites extracted from infected tubers and stems, were analysed as mentioned in section 2.4.5. Under the GC-MS conditions applied in this experiment, the retention time (R_t) for the methylated CFA standard (m/z 222) was identified as 19.87 min (Figure 6.2). GC-MS analysis of the methylated compounds from rotten tubers and stem tissue also confirmed the presence of a peak m/z 222 corresponding to the CFA standard (Figure 6.3). Further mass spectral analysis of the peaks eluted at 19.87 min confirmed the presence of fragment ions m/z 193, m/z 191 and m/z 190 (diagnostic peaks of CFA), similar to the CFA standard (Bender et al., 1989; Mitchell, 1982) (Figure 6.3).

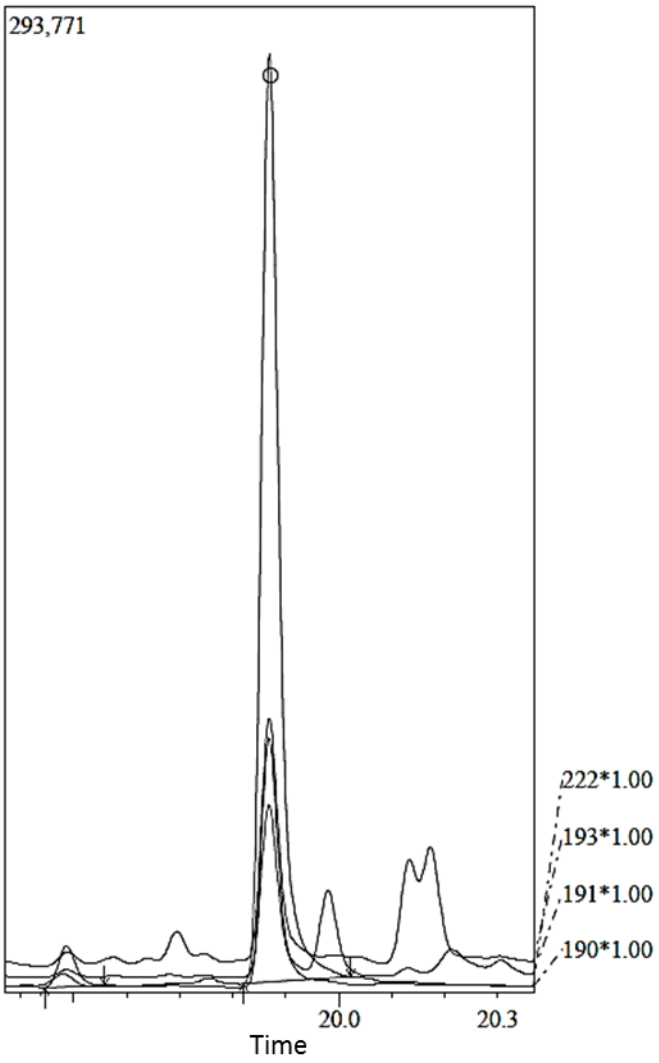


Figure 6.2: GC-MS chromatogram of methylated Coronafacic acid standard purified from *P. syringae* at Rt of 19.87 min.
Peaks corresponding to m/z 222, m/z 190, 191 and 193 (diagnostic ion) were used to characterise Coronafacic acid (Bender et al., 1989).

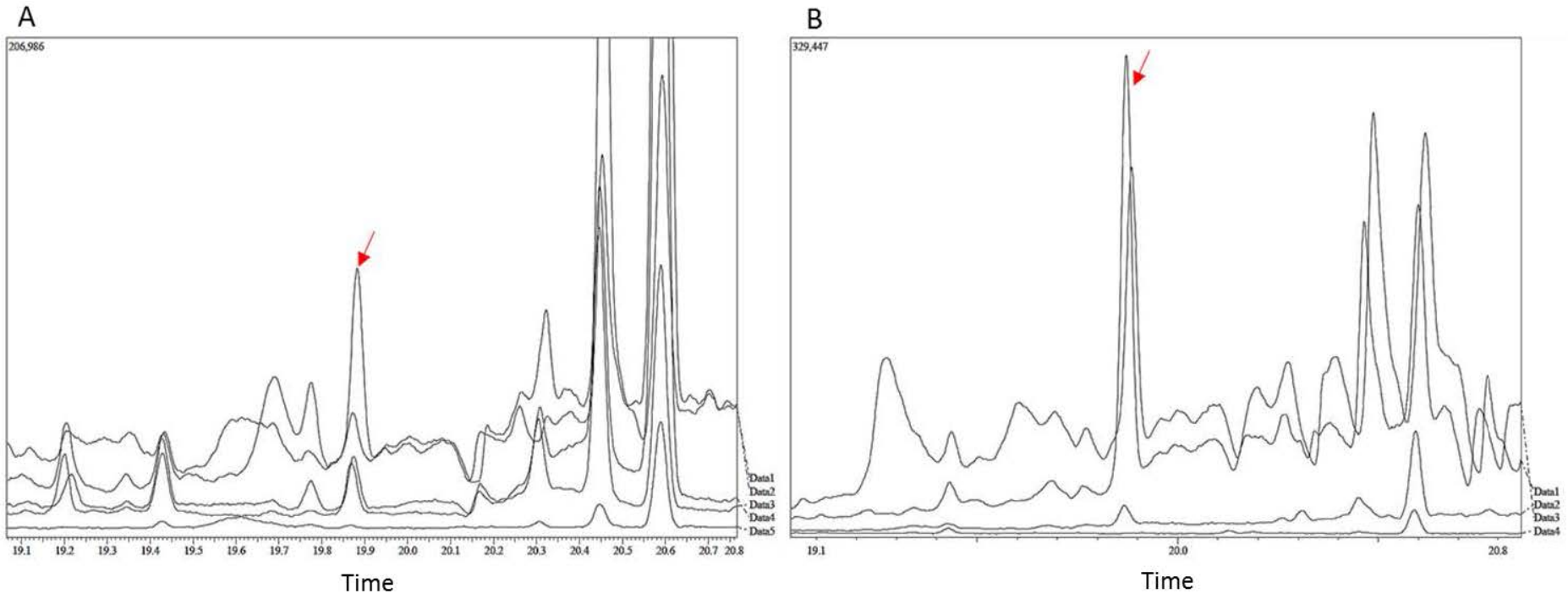


Figure 6.3: GC-MS chromatogram showing peaks corresponding to m/z 222 with Rt of 19.87 min (Red arrow) from metabolites collected from (A) tuber and (B) stem tissue infected with *Pbr* ICMP19477 and *Pba* SCRI1043, respectively.
(A) Data 1 to 4 represent the metabolites collected from individual tubers infected with *Pbr* ICMP19477, and Data 5 represents the metabolites collected from non-inoculated potato tuber. (B) Data 1 to 3 represents the metabolites collected from individual stems infected with *Pba* SCRI1043 and Data 4 represents the metabolites collected from non-inoculated potato stem.

To approximate the amount of CFA produced *in planta* and to verify that the CFA mutant *Pba* SCRI1043ΔHAI2 was not producing the putative phytotoxin, metabolites were subsequently obtained from stem tissue infected with either *Pba* SCRI1043 or *Pba* SCRI1043ΔHAI2 and the metabolites were analysed using GC-MS analysis. Chromatogram analysis of metabolites from the wild-type infected stem tissue confirmed the presence of the characteristic CFA peak m/z 222 at a retention time of 19.87 min corresponding to the CFA standard (Figure 6.4A). No m/z 222 peaks were identified in the extracts obtained from *Pba* SCRI1043ΔHAI2 infected stems (Figure 6.4B) or non-inoculated stems (Figure 6.4C).

The production of CFA was quantified in stems infected with *Pba* SCRI1043, based on the area of the peak m/z 222 (abundance ion) and comparisons with those generated by the CFA standards (Figure 6.6). The relative concentrations of CFA in stems ranged from 8 $x10^4$ to 4.6$x10^5$ (No units) but were highly variable probably due to differences in the severity of infection between samples.

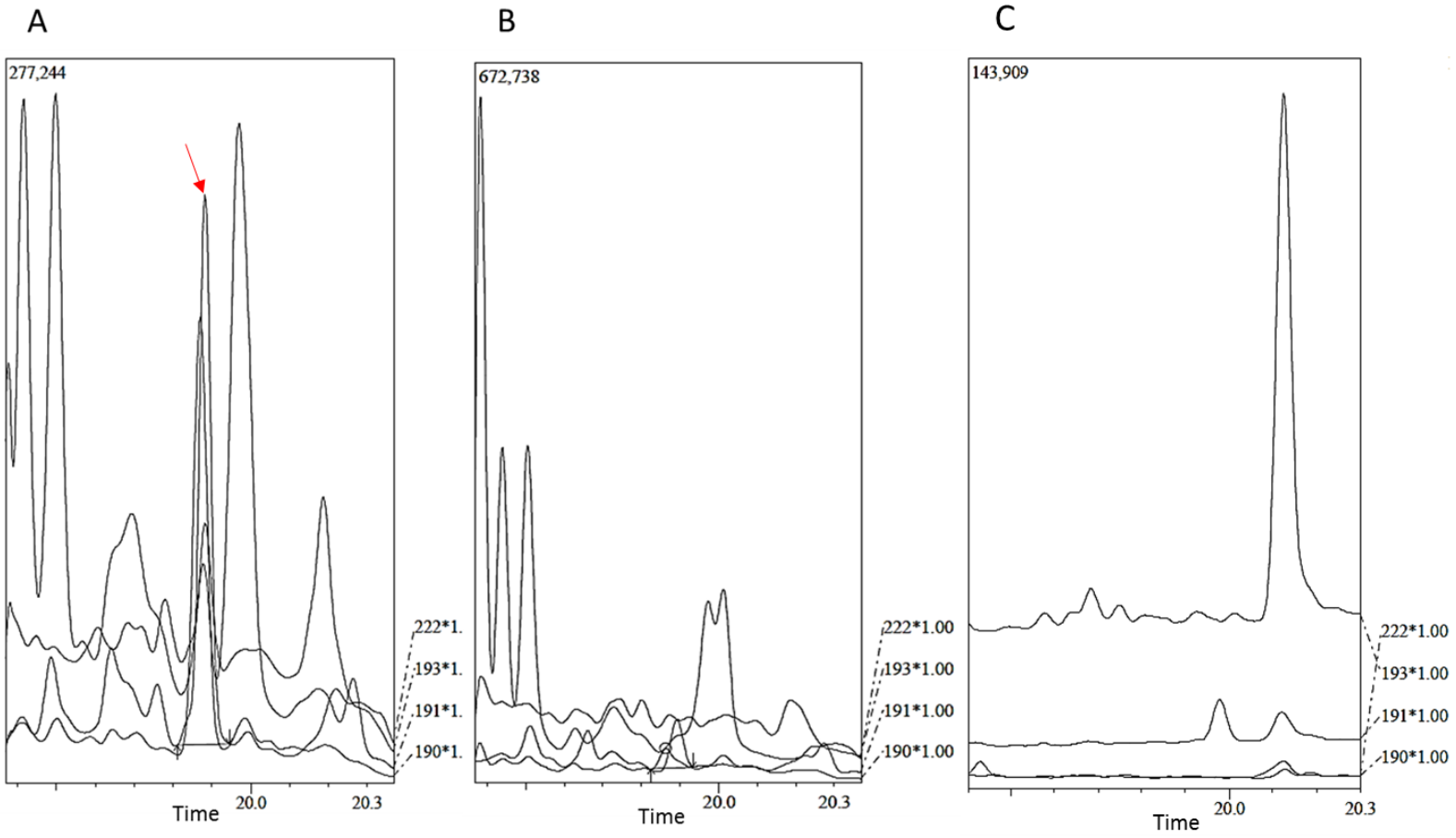


Figure 6.4: GC-MS chromatogram presenting the peaks corresponding to Coronafacic acid m/z 222 with Rt of 19.87 min from metabolites (A) collected from stem tissue infected with *Pba* SCRI1043 (red arrow) (B) collected from stem tissue infected with *Pba* SCRI1043ΔHAI2 (C) collected from non-infected stem tissue.

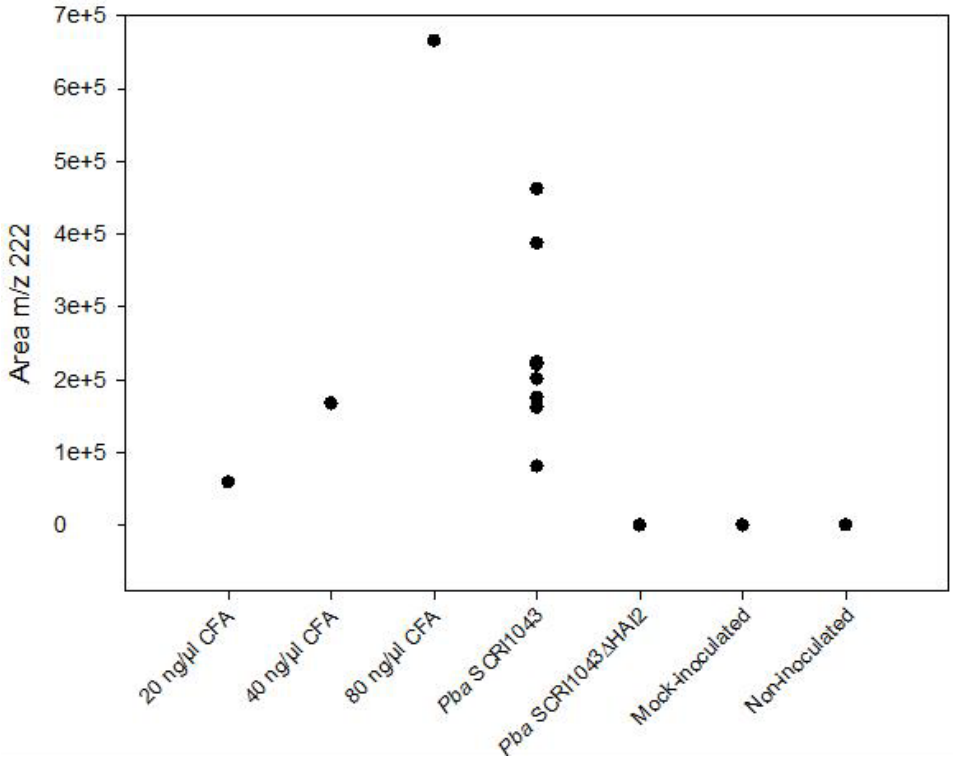


Figure 6.5: Area of peak m/z 222 at Rt 19.87 min, corresponding to Coronafacic acid in *Pba* SCRI1043 and *Pba* SCRI1043ΔHAI2 infected stems.

Note: For *Pba* SCRI1043, each points represents one replicate, while the single points for each of *Pba* SCRI1043ΔHAI2, mock-inoculated and the non-inoculated controls represent the mean area of m/z 222 peak observed across ten replicates.

6.1.3 Detection of CFA-Val *in planta* during infections with *Pectobacterium* spp. using GC-MS

Having confirmed the *in planta* production of CFA and given that the *cfa* biosynthetic gene cluster harbours *cfl*, which is required for the ligation of CFA to an amino acid, GC-MS was used to identify *in planta* production of CFA conjugates. A CFA-Val standard (kindly provided by Robin Mitchel and Dave Greenwood, Plant & Food Research), along with the metabolites extracted from *Pba* SCRI1043 infected potato stems, were analysed as described in section 2.4.5 The retention time of methylated CFA-Val (m/z 321) was identified as 27.28 min (Figure 6.6). Though the major ion corresponding to the fragment m/z 321 was not observed in *Pba*

SCRI1043 infected stem tissue, the fragment ions m/z 191 (most abundant ion in CFA-Val) (Bender et al., 1989), m/z 190 and 163 corresponding to the CFA-Val standard were identified at 27.28 min (Figure 6.7A). The failure to observe a peak at m/z 321 could possibly be due to the low abundance of the fragment ion m/z 321. No peaks relating to m/z 321 or m/z 191 (corresponding to CFA-Val standards) were identified in the methylated volatiles obtained from *Pba* SCRI1043ΔHAI2 infected stems (Figure 6.7B) or non-inoculated plants. Thus, this preliminary study provided the first evidence for *in planta* production of a CFA-Val conjugate.

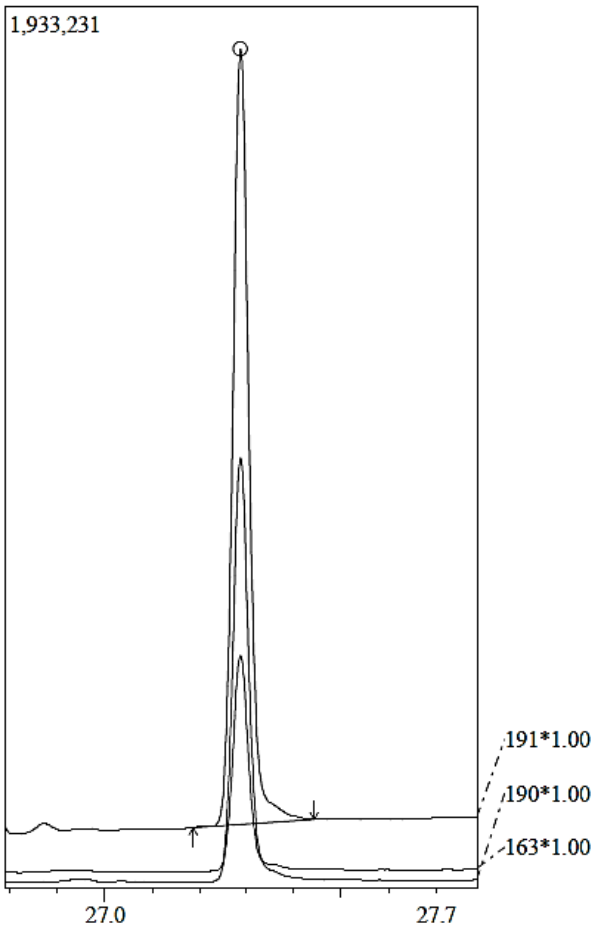


Figure 6.6: GC-MS chromatogram of methylated Coronafacic acid-Valine standard purified from *P. syringae* with Rt of 27.28 min.

Peaks corresponding to m/z 191 (diagnostic ion), m/z 190 and m/z 163 were used to characterise CFA-Val (Bender et al., 1989).

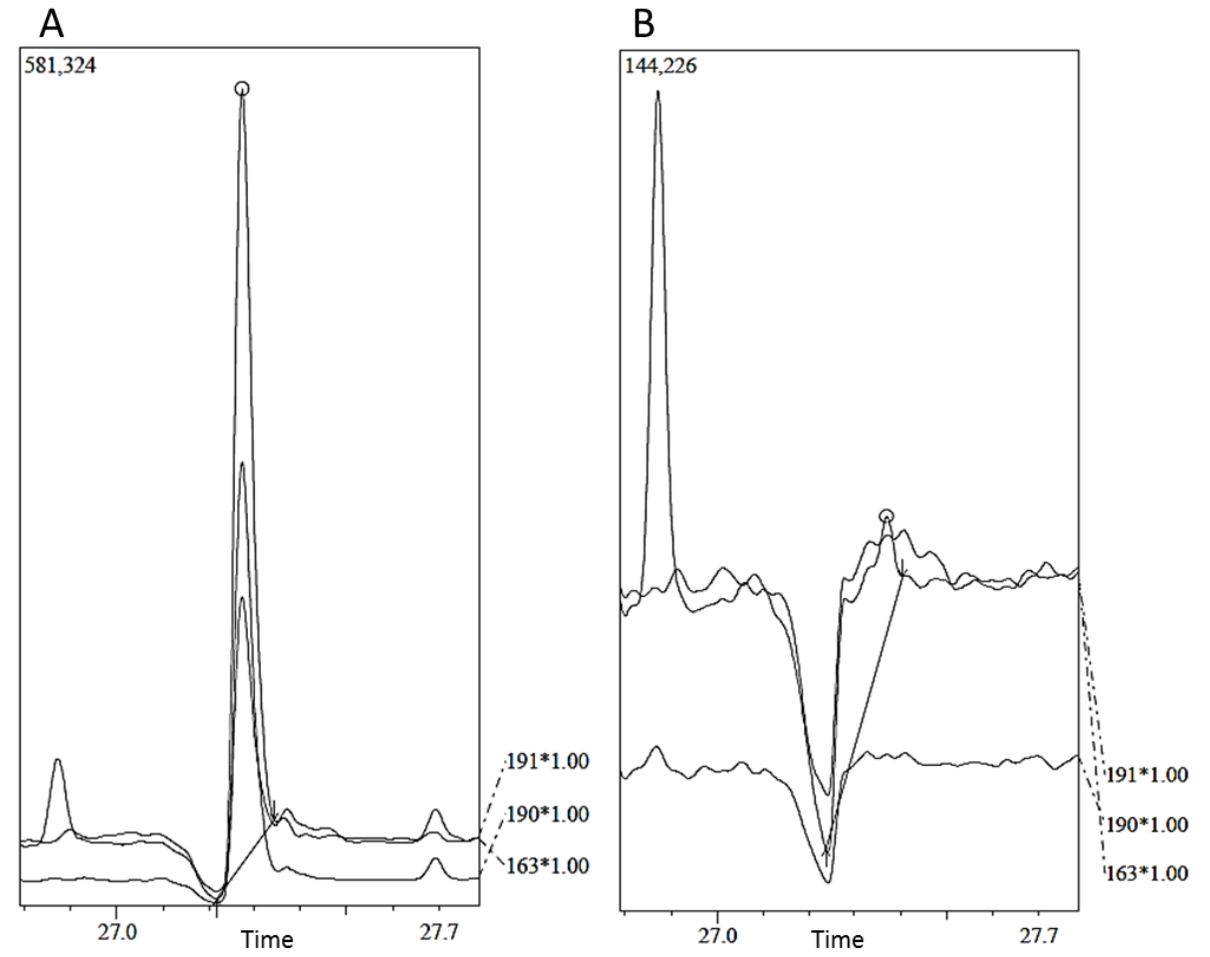


Figure 6.7: GC-MS chromatogram presenting the peaks corresponding to Coronafacic acid-Valine m/z 191 with Rt 27.28 min from metabolites (A) collected from stem tissue infected with *Pba* SCRI1043 (B) collected from stem tissue infected with *Pba* SCRI1043ΔHAI2.

6.1.4 *In vitro* production of CFA

The quantities of the CFA-Val conjugate produced *in planta* were too low for further purification and quantification. Furthermore, purification of the CFA and the CFA conjugates from infected tissue was disadvantageous due to the presence of other plant metabolites that could hinder the purification process. Thus, *in vitro* culture conditions were investigated for further CFA and CFA-Val purification.

As part of trying to establish an *in vitro* system, *Pba* SCRI1043 was cultured in 50 ml cultures of MM medium supplemented with either sucrose, fructose or glucose. In addition to sugars,

MM media was also supplemented with M9 salts and either 5% v/v stem or tuber extract as described in section 2.1.1. *In vitro* transcription of the CFA biosynthetic cluster and biosynthesis of the product were subsequently examined using qRT-PCR and GC-MS analysis, respectively.

Expression of *cfa6* in media supplemented with plant extract

In vitro induction of the *cfa* biosynthetic genes was attempted by supplementing 5% v/v stem extract to the MM media. However, due to the nature of the extract, the media could not be sterilised using the ultra-centrifugation method described in section 2.1.1 (data not shown). Due to contamination issues, this approach was not investigated further.

Expression of *cfa6* in media supplemented with tuber extract

The strongest difference in *cfa* gene expression was related to the growth phase of *Pba* SCRI1043. On average the *cfa6* quantities were lowest at 24 hpi, rising noticeably after 30 hpi and rising again at 48 hpi ($p = 0.010$). No strong evidence for a difference in *cfa* expression relating to different sugars was observed ($p > 0.2$). Furthermore, the normalised $\log_{10}$ quantities of *cfa6* expression in media with neither salt, nor tuber extract were lower when compared to the media supplemented with either salt, or tuber extract, or both. There was some evidence of the influence of salt and plant extract on the expression of *cfa6* ($0.1 > p > 0.05$ for the salt by tuber extract interaction, across *cfa6* gene expression) (data not shown), however, no significant induction of the *cfa* biosynthetic cluster was observed in media supplemented with both salt and tuber extract.

Analysis of the *in vitro* production of CFA and CFA conjugates in the metabolites extracted from *Pba* SCRI1043 grown in MM medium supplemented with either M9 salts or M9 salts and tuber extract showed no peaks relating to the mass spectra and retention time of the CFA standard (data not shown).

6.4 Discussion

To date, the role of CFA in the virulence of SRE has only been confirmed by studies of *Pba* SCRI1043 in potato stems (Bell et al., 2004; Panda et al., 2016), and the effect of CFA on infection of the tuber has not been investigated. This seems a major oversight given that SRE primarily infect potato through the lenticels and wounds in tubers (Perombelon, 2002; Perombelon & Hyman, 1989; Perombelon & Kelman, 1980), and as a result the tuber would be the first plant organ to elicit a response to the pathogen. In this study, after observing no differences in the symptoms in tubers caused by inoculation with a CFA mutant and the wild-type (Chapter 3), the possibility of tissue-specific transcription of the CFA biosynthetic cluster was investigated by qRT-PCR. The results of these experiments (presented in the Table 6.4) indicated that the *cfa* gene cluster is highly transcribed in bacterial populations infecting stems compared to those in tubers, supporting the observed differences in the impacts of CFA on the outcome of the interactions between the pathogen and its host in these two tissues. Such tissue-specific outcomes, suggest that the importance of CFA and other virulence factors might be dependent on the phase of infection. Although studies of human pathogens have begun to dissect the coordinated expression of virulence factors during different stages of host infection, few such studies have been conducted on plant pathogenic SRE. Thus, studying the coordinated expression of virulence factors during the various stages of colonisation (including both latent and pathogenic phases) would provide greater understanding of the mechanisms used by the SRE to infect their plant hosts.

The fact the *cfa6* gene expression was highly induced only in stems, suggests that plant metabolites may be responsible for the induction of CFA production. Studies have previously shown increased mobilization of HAI2 and induction of the *cfa* gene cluster in *Pba* SCRI1043 during blackleg infection (Vanga et al., 2012). Although the correlation between these two phenomena remains unknown, mobilisation was predicted to result from the presence of unknown plant metabolites or stress-related signals imposed by the plant environment (Vanga et al., 2012). In addition, studies in *S. scabies* to characterise COR-like metabolites identified that the *cfa1* promotor, known to regulate the CFA gene expression, was induced during the

colonization of the plant root by *S. scabies* 87-22 (Fyans et al., 2015). Further, the plant metabolite-controlled *cfa* expression and production could be studied by using other alternative approaches including monitoring the CFA transcription in the apoplastic fluid supplemented *in vitro* cultures and analysing the plant metabolites in the apoplastic fluid. As an alternative, the plant metabolites in the infected tissues could be characterised to identify the inducer molecule of *cfa* expression.

In addition to the tissue specific expression of *cfa* gene, the most significant finding to emerge from this study was the detection of both CFA and CFA-Val *in planta* (stems) during blackleg infection by *Pba* SCRI1043 (Figures 6.4 and 6.7). Tentative evidence of CFA production by *Pbr* was also shown in tubers (Figure 6.3). This was the first time CFA production was detected *in planta*, as previous studies in *P. syringae* and *S. scabies* have always examined CFA production under *in vitro* conditions (Fyans et al., 2015). The importance of this finding should not be underestimated, as the absence of a CMA biosynthetic cluster in *S. scabies* and pectobacteria has raised questions as to the need for a conjugate for bioactivity of CFA and the identity of the conjugate. Mutation studies in *P. syringae* and *S. scabies* have shown that the *cfl* gene is required for the ligation of CFA to an amino acid to form coronafacoyl amide (Fyans et al., 2015; Rangaswamy et al., 1998). In addition, studies have also reported that conjugation of CFA to amino acids is essential for the bioactivity in plants (Brooks et al., 2005; Fyans et al., 2015; Uppalapati et al., 2005). Here, we showed that CFA is present in the plant upon infection with *Pba* SCRI1043 and that the concentration 7 dpi is approximately 20ng/µl to 80 ng/µl. This confirmed that the CFA biosynthetic cluster was not only transcribed, but was also translated into a final product in potato. Furthermore, quantification of CFA provided evidence of the concentration produced by the bacterium in the host, enabling these concentrations to be used in future studies, to provide a realistic idea of the role of CFA in the host.

Due to the lack of availability of other CFA conjugates as standards, production of other possible *N*-coronafacoyl amides during infection cannot be confirmed. In addition, when compared to *Pba* SCRI1043, the *cfl* gene involved in the ligation was highly variable between the species with 93% amino acid identity for *Pcc* UGC32, 35% in *Pba* ICMP1526 and 31% in *Pbr* ICMP19477 (Panda et al., 2016). Given this variability, further studies need to be carried out

to characterise the CFA conjugates produced by *Pectobacterium* spp. during blackleg and soft rot infection in potato. This would also provide an insight in to the tissue specific expression of CFA conjugates. To confirm the role of these conjugates during pathogen infection, the interaction of CFA conjugates with COI1, the key receptor of JA-Ile in JA signalling pathway should be studied. Furthermore, the interactions between CFA conjugates and the other defence-related TFs and complex should be studied to understand the broader impact of CFA-conjugates on host defence.

6.5 Conclusion

This study provides an insight into tissue specific CFA and CFA-Val production in potato plants during *Pba* SCRI1043 infection. Methods developed during this study such as the extraction of the methylated CFA and CFA-Val from infected plant tissues, will prove valuable and will enable further studies on other CFA conjugates produced by *Pectobacterium* spp. during infection. Further work including purification of CFA and related conjugates from *in planta* and *in vitro* will be useful to understand the *in planta* substrate specificity of the CFL enzyme.

Chapter 7 General Discussion

The work in this thesis was conducted to understand the potato-*Pectobacterium* interaction and to provide an insight into the molecular strategies employed by the host against these pathogens during the necrotrophic stage of infection. Additionally, the effect of CFA on the host defence system was investigated. Given the dual biotrophic and necrotrophic lifestyle of pectobacteria and the absence of CMA (required for the production of COR, a phytotoxin) it was suggested that the role of CFA in the virulence of the pathogen might differ in *Pectobacterium* taxa. Overall, the results of this study provides insight into promising potato genes to target for further studies on resistance interactions and for future resistance breeding programmes.

7.1 Response of potato tuber to pectobacteria

Initially, the study aimed at confirming the varying degree of aggressiveness between the pectobacteria strains on the potato tubers tissue. The pathogenicity assay conducted in this study confirmed the aggressiveness of *Pbr* ICMP19477. In addition, the transcriptome dynamics suggested that when compared to *Pba* SCRI1043 the magnitude of the fold change observed in response to *Pbr* ICMP19477 was significantly higher. Given the recent observation of the ubiquitous nature and confirmed aggressiveness of this taxon on host plants, the results observed in this study was not surprising. However, as the varying degree of aggressiveness between *Pba* SCRI1043 and *Pbr* ICMP19477 strains suggest, the pathogen induced virulence on the host tissue depends on additional virulence factors, possibly acquired during the course of evolution. Though comparative genomics between *Pba* SCRI1043 and *Pbr* ICMP19477 strains have successfully identified the difference in the virulence determinants (Panda, 2014), the role of additional virulence determinants acquired by *Pbr* ICMP19477 during their pathogenicity and the host induced response to these virulence determinants in potato tubers still remains to be studied. For example, *Pbr* ICMP19477 encodes the T3SS effector proteins

resembling the *srfABC* T3SS-associated gene cluster of *Salmonella* species (Lostroh & Lee, 2001) and *P. syringae* (Petnicki-Ocwieja et al., 2002). Secretion studies in *P. syringae* have also confirmed the Hrp-mediated secretion of *srfC* (Petnicki-Ocwieja et al., 2002). Identification of a putative T3SS effector protein in *Pbr* ICMP19477 suggests their possible role in the virulence of this bacterium. However, further knock-out, pathogenicity and transcriptional studies are required to confirm the Hrp-mediated secretion, their role in the virulence of *Pbr* ICMP19477 and the host mediated defence response to this effector protein.

7.1.1 Transcriptional dynamics in response to pectobacteria

7.1.1.1 DAMP-mediated defence response in potato

Pectobacterium infection activates several defence related pathways in 'Summer Delight' potato tubers. Redox and calcium-mediated signalling, plant hormones, secondary metabolites and transcriptional factors were all DE in potato tubers during *Pectobacterium* infection. Similar patterns of gene expression observed in response to mock-inoculation, pectobacteria infection and exogenous CFA showed that mechanical damage and pathogen attack induce the same defence pathways. *Pectobacterium* spp. secrete a range of PCWDEs which are capable of degrading the plant cell wall sugars resulting in cell leakage (Toth et al., 2003). Consequently, the cell wall fragments are perceived by PRRs resulting in downstream defence signalling (Dodds & Rathjen, 2010; Jones & Dangl, 2006). Likewise, the mechanical damage of plant tissue results in the damage to the cell wall, thus activating the "damage to self" signals and consequent downstream defence related signalling.

The evidence supporting the PCWDEs and the related activation of DAMP-mediated PTI in potato tubers during pectobacteria infection is considerable. Genes related to several receptor kinases were DE in response to pectobacteria at 24 hpi (Table 4.2). In *Pectobacterium*, the presence of PCWDEs and their role in the virulence of this pathogen has long been appreciated. PCWDEs in pectobacteria are regulated in a population-density dependent manner, also known as quorum sensing, thus preventing the premature release of

the PCWDEs and consequently the DAMP-mediated PTI (Liu et al., 2008). Differential expression of the receptor kinase and the activation of the other defence pathways is consistent with the DAMP-mediated PTI, where the pathogen releases PCWDEs after reaching certain population density, thus resulting in activation of defence responses. In these experiments, the induction of significant defence response occurred at 24 hpi, when the population density was between 10^8-10^9 (based on the *in planta* growth dynamics analysis). In addition, the induction of plant cell wall modification genes along with induced expression of cellulose synthase (Table 4.2), suggest host mediated cell wall synthesis and cell wall modification. Cell wall modification, or cell wall thickening, is induced in plants when the plant cell wall is degraded by the action of PCWDEs, where the plant tries to restrict bacterial entry by synthesising the secondary cell wall (Malinovsky et al., 2014). Thus the perception of the DAMP signals results in the induction of cell wall synthesis and modification which acts as a first layer of plant defence against the invading pathogen (Malinovsky et al., 2014).

Calcium- and ROS-mediated defence response in potato tubers

The major genes involved in the early DAMP-mediated PTI were those of the PI-PLC pathway, Ca^{2+} binding and redox related genes. Phospholipase and phospholipase-derived molecules in plants are involved in the early stages of host response during PAMP and DAMP-mediated PTI. They play a crucial role in inducing defence response through their activation of Ca^{2+} signalling, modulation of ROS, activation of MAPK and defence related gene activation (Chen et al., 2007; Munnik, 2014; Vossen et al., 2010). Studies to confirm the interaction of JAZ with COI1 have identified inositol pentakisphosphate, a derivative of PI-PLC pathway as the critical component of the co-receptor complex (Sheard et al., 2010). Consistent with the induction of *InsPk*, the gene involved in the metabolism of inositol-1,4,5-triphosphate-5-phosphatase to inositol pentakisphosphate, and the involvement of inositol pentakisphosphate in the JA-Ile receptor complex suggests that the induction of PI-PLC6 pathway during the DAMP-mediated PTI plays a significant role in downstream activation of defence response. Previously, the PI-PLC pathway has been associated with drought stress in different tissues of potato, including tubers (Canonne et al., 2011).

Cytosolic Ca^{2+} influx has been associated with the early physiological changes associated with pathogen infection (Zipfel, 2009). In this study, infection of potato tubers, mock-inoculation and treatment with exogenous CFA resulted in the induction of Ca^{2+} binding proteins, suggesting a DAMP-mediated Ca^{2+} influx (Ju et al., 2012; Tsuda & Katagiri, 2010). However, induction of the PI-PLC pathway also results in Ca^{2+} influx. Consistent with the induction of PI-PLC6 pathway it is tempting to suggest that the observed induction of Ca^{2+} binding proteins are a result of PI-PLC6 mediated cytosolic Ca^{2+} influx. However, further molecular, genetic and biochemical studies are required to confirm the PI-PLC mediated Ca^{2+} influx in potato tubers during potato-*Pectobacterium* interactions.

Genes involved in oxidative burst in plants, including genes annotated as peroxidase, were induced during *Pectobacterium* infection, mock-inoculation and in response to exogenous CFA. This suggests a DAMP-mediated induction of ROS. Peroxidase genes in plants are responsible for the generation of the oxidative burst (O'Brien et al., 2012), one of the early physiological changes related to PAMP/DAMP-mediated plant defence response (Galletti et al., 2009; Spoel & Loake, 2011). In addition to peroxidase, glutaredoxin and thioredoxin (the two genes involved in the modulation of ROS) were significantly induced across all treatments. Previous studies in *Arabidopsis* have confirmed the roles of these genes in the downstream activation of *PR1* expression and SA-JA antagonistic interactions, respectively (Herrera-Vásquez, Carvallo, et al., 2015; Ndamukong et al., 2007). Consistent with the induction of these ROS modulating genes, genes encoding *PR1* were significantly induced across all treatments. Though *PR1* has been historically associated with the induction of SA-mediated defence response, in this study, genes related to SA biosynthesis were not DE; neither in response to pectobacteria nor to mock-inoculation. In tobacco, benzoate has been identified as the precursor of SA biosynthesis, while in *Arabidopsis*, isochorismate is the immediate precursor of SA biosynthesis (Tada et al., 2008). However, the induction and the regulation of SA in potato tubers remains to be elucidated. Various studies have previously confirmed the ROS-mediated induction of SA pathway (Baxter et al., 2014). Taken together, given the lack of SA signalling pathway, the induction of *PR1* observed in this study is suggested to be a ROS-mediated SA signalling. However, further knockout studies are required to confirm the role of

glutaredoxin and thioredoxin in the potato SA pathway and subsequent *PR1* expression in potato tubers.

Phytohormone-mediated crosstalk in potato tubers

Traditionally, phytohormones have been associated with plant innate immunity. Various studies have confirmed crosstalk between phytohormones, and the role of these complex mechanisms in deploying active defence responses based on the pathogen's lifestyle (Pieterse et al., 2009; Pieterse et al., 2012). In this study, differential expression of genes involved in phytohormone biosynthesis and signalling were observed in response to mock-inoculation, pectobacteria and exogenous CFA.

Of particular interest, genes involved in the ET biosynthesis were induced across all treatments. Previously, ET-mediated signalling in plants has been associated with generic defence response against various pathogens and wounding (Broekaert et al., 2006; Merchante et al., 2013; Wang et al., 2002). Additionally, ERFs also play a vital role in fine tuning the antagonistic and synergistic interaction between other phytohormones including JA and SA-mediated defence response (Lorenzo, Piqueras, Sanchez-Serrano, et al., 2003). In this study, consistent with ET biosynthesis, genes related to the ET signalling pathway (including ET receptors and ERFs) were induced in response to pectobacteria and mock-inoculation. Thus, the differential expression observed in this study suggests that the ET biosynthesis and signalling pathway in tubers may be linked to the generic defence response induced by tubers against mechanical damage and pathogen attack.

In addition to differential expression of the ET signalling pathway, genes annotated as chitinase and endochitinase were induced in response to both pectobacteria, exogenous CFA and mock-inoculation. However, the magnitude of this induced expression was consistently higher in response to pathogen treatments, than that observed in response to wounding. Other studies have also observed that the induction of necrosis in plant tissues induces the accumulation of various PR proteins including SA inducible PR1, JA and ET inducible chitinase,

defensin and PR4 (hevein like proteins) (Glazebrook, 2005; Pieterse et al., 2006; Pieterse et al., 2009; Pieterse et al., 2012). The observed differential expression of chitinase in response to pectobacteria and mock-inoculation suggests DAMP-mediated induction.

Interestingly, genes related to JA biosynthesis, a key pathway related to bacterial defence, was not DE in response to *Pectobacterium* spp. Alternatively, the expression of *des* (a gene involved in the synthesis of colneleic and colnelenic acid from 9-hydroperoxy linolenic acid) was significantly induced in response to pectobacteria and wounding. Consistent with previous research (Fammartino et al., 2007; Howe & Schilmiller, 2002; Stumpe et al., 2001; Weber et al., 1999), induction of these genes suggest they have a significant role in antimicrobial activity against pectobacteria, as well as initial defence response against mechanical damage. Further studies are required to confirm their antimicrobial activity against SREs. In addition, coordinated biosynthesis of JA and ET and consequent induction of JA-mediated ERF1 activation is required for the synthesis of *defensin* (*PDF1.2*), an antimicrobial compound required for active defence against necrotrophic pathogens (Glazebrook, 2005; Mengiste, 2012). In agreement with the lack of the activation of the JA biosynthesis pathway, the genes responsible for *defensin* were significantly down regulated across pectobacterial treatments.

The lack of induction of JA biosynthetic genes, shows that the defence response against pectobacteria in 'Summer Delight' potatoes is JA-independent. In plants, 13-hydroperoxy linolenic acid are usually involved in the JA biosynthesis (Blée, 2002). It is hypothesised that the varying composition of the 9- and 13-hydroperoxy linolenic acid determines the JA biosynthesis and thus the active defence response induced by the host to invading pathogens. Further studies comparing the hydroperoxides of linolenic and linoleic acid and the expression of JA biosynthetic pathway between the susceptible and the resistant cultivar will provide valuable insight into the host resistance mechanism against pectobacteria.

During pathogen attack, deployment of resources into defence responses is imperative for plant survival. However, when the plant channels its energy towards defence, it therefore must compensate by taking energy/resources away from development processes. In

Arabidopsis mutants with constitutive expression of defence genes displayed stunted growth and reduced fertility in contrast to mutants defective in defence responses, which were taller (Heil & Baldwin, 2002). Thus, in order to establish a favourable energy balance, up regulation of defence related genes results in the down regulation of growth related hormones such as GA (Huot et al., 2014). The down regulation of the GA biosynthesis genes and induced expression of DELLA (negative regulator of GA), reported in chapter 4, is consistent with this hypothesised trade-off between plant growth and defence.

Sn-1 and Sn-2 two genes encoding antimicrobial peptides in potato were significantly induced in response to *Pbr* ICMP19477. Previous studies have confirmed the role of these genes in active defence against pectobacteria (Almasia et al., 2008; Mohan et al., 2014; Nahirñak et al., 2012). The differential expression observed in response to pectobacteria, exogenous CFA and mock-inoculation suggest the lack of bioactive GAs, however snakin genes (also knowns a GASA proteins) have been identified as GA-regulated proteins (Nahirñak et al., 2012). Thus the regulation of these antimicrobial peptides remains to be studied.

Taken together, the induced expression of diverse PR genes in response to pectobacteria suggests the activation of diverse defence responses against the invading pathogen. Further characterisation of phytohormone levels during plant infections could confirm the lack of JA and GA in tubers during this response, and the possible role of DELLA (inhibitors of GA) in maintaining the balance between the defence and development in potato tubers.

Sesquiterpene-mediated defence in potato tubers

The final group of genes involved in the DAMP-mediated defence response in potato tubers is that of secondary metabolism related genes. Genes related to the terpenoid biosynthesis were DE in response to pectobacteria, exogenous CFA and mock-inoculation (though the DE was non-significant induction during this last treatment). Previously, sesquiterpene in potato have been identified to be involved in the defence response against *P. infestans* (Coxon et al., 1979; Katsui et al., 1972; Zook & Kuć, 1991). The differential expression of genes involved in

the terpenoid biosynthesis pathway, shows that the production of secondary metabolites during DAMP-mediated PTI play an influential role in potato-*Pectobacterium* interactions. Further chromatography studies and anti-bacterial assays are required to characterise the exact compounds produced during potato-*Pectobacterium* interactions and thus their role as an antimicrobial compound during these interactions.

Collectively, the differential expression of various pathways and their gene categories revealed that DAMP-mediated PTI plays a significant role during potato-*Pectobacterium* interactions. Having identified one effector in pectobacteria and given that ETI-mediated HR and subsequent cell death favour the necrotrophic phase of bacterial infection, this research shows that the observed DAMP-mediated defence response provides the first layer of defence against this pathogen. Furthermore, induction of several defence related pathways over the duration of the experiment reveals that the early defence response in potato tuber to *Pectobacterium* infections requires the induction of several different pathways and antimicrobial genes. Therefore, based on the results from this study, we propose a potato-*Pectobacterium* early defence response model, is dependent on DAMP-mediated PTI (Figure 7.1). This includes the perception of damage signals, activation of the PI-PLC signalling cascade, redox-mediated SA signalling, activation of ET biosynthesis, activation of downstream transcriptional reprogramming and biosynthesis of secondary metabolite and antimicrobial products.

The model presented here provides a comprehensive view on the different defence responses deployed by the host to a pathogen. These insights will prove valuable to understand the defence strategies deployed by potato tuber 'Summer Delight' to other seed borne pathogens, including *P. infestans* and *S. scabies* to name a few. In addition, the gene clusters observed in this study will be useful as candidate marker genes for further transcriptional analysis on other resistant cultivars. Given the main objective of this study was to identify the organ specific defence response to SREs, the defence response observed in tubers will serve as a benchmark for future studies to compare the defence response to these SREs in stems.

7.2 Response of potato tubers to CFA

The second key objective of this thesis was to identify the role and the molecular interaction of CFA, a phytotoxin required to manipulate phytohormone-mediated defence responses, with potato tubers during *Pectobacterium* infections. To study the impact of CFA on pectobacteria pathogenicity in potato tubers, the virulence of *Pba* SCRI1043ΔHAI2 and *Pba* SCRI1043 were compared. Interestingly no significant impact of HAI2/CFA was observed during soft rot infection of tubers. However, previously, CFA was shown to play a considerable role in the virulence of *Pba* SCRI1043 during blackleg infections (Panda et al., 2016). The results observed in this study was in agreement with the *in planta* and *in vitro* CFA expression (conducted later in this research). Though the expression of the *cfa* gene cluster was induced during both soft rot and blackleg infections, the magnitude of induced expression observed in the stem was significantly higher than the induction observed in tubers during soft rot infection. This suggests that the plant metabolites present in the stem play a significant role in induced expression of CFA. Alternatively, given the organ specific defence response in plants and the previously known role of COR in manipulating host defence response it is also hypothesised that the concentration of CFA required to manipulate host defence response may be significantly higher in stems than in tubers.

7.2.1 CFA/CFA-conjugate mediated host defence manipulation in potato tubers

The transcriptional dynamics observed in response to *Pba* SCRI1043ΔHAI2 (when normalised with *Pba* SCRI1043), *Pba* SCRI1043 and *Pbr* ICMP19477 suggested CFA-mediated *MYC2* activations. However, the induced expression of *MYC2* genes in response to the wild-type, contradicts the lack of JA biosynthesis pathway, as inferred through transcriptomic analysis, and suggests a JA-independent *MYC2* activation. Infection of potato tubers by the HAI2 mutant resulted in down regulation of the gene encoding *MYC2*. Previous studies to

understand the COR-mediated host defence response manipulation in tomato and *Arabidopsis* confirmed the interaction of COR with the COI1-JAZ protein complex, resulting in the activation of MYC2-mediated JA signalling pathway (Katsir et al., 2008; Melotto et al., 2008; Zheng et al., 2012). The JA-independent induced expression of the genes encoding *MYC2* and *JAZ*, and the consistent down regulation of *MYC2* in response to the HAI2 mutant, suggests that CFA interacts with the COI1-JAZ complex resulting in the activation of an inappropriate defence response in potato tubers during *Pectobacterium* infections. Studies to understand the JA signalling and the COR mediated JA signalling have also confirmed that JA-Ile, the bioactive jasmonate was required to interact with the COI1-JAZ complex (Thines et al., 2007). Consistently, the ability of COR to manipulate the JA signalling pathway is primarily due to it being a structural mimic of JA-Ile (Geng et al., 2014).

Treatment of potato tubers with exogenous CFA resulted in induced expression of the gene encoding *JAR1* which is required for the conjugation of JA to the amino acid. Previous studies in tomato have also confirmed that conjugation of CFA to amino acid is required for the biological activity of CFA (Uppalapati et al., 2005). Furthermore, recent studies in *S. scabies* have also shown that CFA-Ile, but not unconjugated CFA, induces root elongation in radish seedlings, confirming the biological activity of CFA-Ile (Fyans et al., 2015). Given that the COR mediated induction of *MYC2* is related to its being a structural mimic of JA-Ile, and the induction of *JAR1* in response to exogenous CFA, it is suggested that not CFA but the CFA conjugate to amino acid is required for the host defence manipulation. Furthermore, secretion of CFA during *Pectobacterium* infection was successfully detected with GC-MS analysis of the infected plant material, confirming the production of CFA and CFA-Val, a CFA-amino acid conjugate.

Identification of free CFA *in planta* (stems) during *Pba* SCRI1043 infection suggests that, like JA biosynthesis, which is based on the feed forward mechanism, CFA biosynthesis in pectobacteria requires free CFA to induce the biosynthetic process. However, the regulators of *cfa* biosynthesis in pectobacteria remains to be studied. Furthermore, having confirmed the variability of *cfl* gene in pectobacteria strains, it is suggested that there will be variations

between the CFA-conjugates produced by *Pectobacterium* taxa. Further, mutation studies in pectobacteria is required to understand the CFA-conjugation process during *in planta* infection and to further understand the role of various CFA-conjugates in the COI1-JAZ interaction and manipulation of the host defence response.

Detection of CFA-Val, a CFA conjugate during *Pectobacterium* infection is a significant result in this study. Studies to date have identified CFA as a virulence factor, however the molecular mechanism and its relevance as virulence determinant during soft rot infection has remained unclear. Taken together, the results from the pathogenicity study, CFA expression, transcriptional dynamics observed in response to HAI2 mutant and exogenous CFA and GC-MS analysis, it is clear that though CFA has an insignificant impact on the virulence of the pathogen during soft rot infection, CFA does play a crucial role in manipulating the defence response of the host. However, given the lack of JA biosynthesis and the pre-existing susceptibility of this cultivar to the *Pectobacterium,* it is suggested that CFA may play a less important role in rendering the particular cultivar 'Summer Delight' susceptible to soft rot infection. It is speculated that CFA may play a more crucial role in the pathogen-mediated host susceptibility in plant tissues with active JA biosynthesis. In summary, this study provides an insight into the manipulation of the defence system of potato by *Pectobacterium* through activating an inappropriate and mistimed defence response in potato tubers by CFA-conjugate mediated *MYC2* induction. Further chromatography and structural studies are required to identify the full spectrum of CFA conjugates during soft rot and blackleg infection and their role in COI1-JAZ interaction.

7.3 Conclusion

Pbr ICMP19477, when compared to *Pba* SCRI1043 was identified as an aggressive pathogen. Despite the variation in the aggressiveness between the pectobacteria strains, the host induced defence response remains to be specific to *Pba* SCRI1043 and *Pbr* ICMP19477. Though

Pectobacterium spp. carry effectors, PCWDEs-mediated DAMP defence appears to be the major response induced by the host against pectobacteria. Redox and Calcium mediated signalling along with phytohormones play a crucial role in inducing an active defence response in potato tubers 'Summer Delight'. *Sn-1* and *Sn-2*, two previously identified genes involved in active defence against pectobacteria, are involved in defence against *Pbr* ICMP19477. Sesquiterpene also play a role in the potato defence response, however the precise compound produced during potato-*Pectobacterium* interaction remains to be characterised. CFA /CFA-conjugate with an amino acid was identified to manipulate host defence response in a *MYC2*-dependent manner, during susceptible potato-*Pectobacterium* interaction. In addition, chromatography studies confirmed the secretion of CFA and CFA-Val during *Pectobacterium* infection in stems. The results from this study provides valuable insight into the potato tuber induced defence response to *Pectobacterium* infection and the role of CFA during potato-*Pectobacterium* interaction. Future studies on the potato-*Pectobacterium* interaction in resistant cultivar and a comparison between the tuber and stem induced defence response will prove valuable for future resistance breeding. Further studies on the interaction of CFA-conjugate and COI1-JAZ complex will provide valuable insight on CFA-conjugate-host interaction.

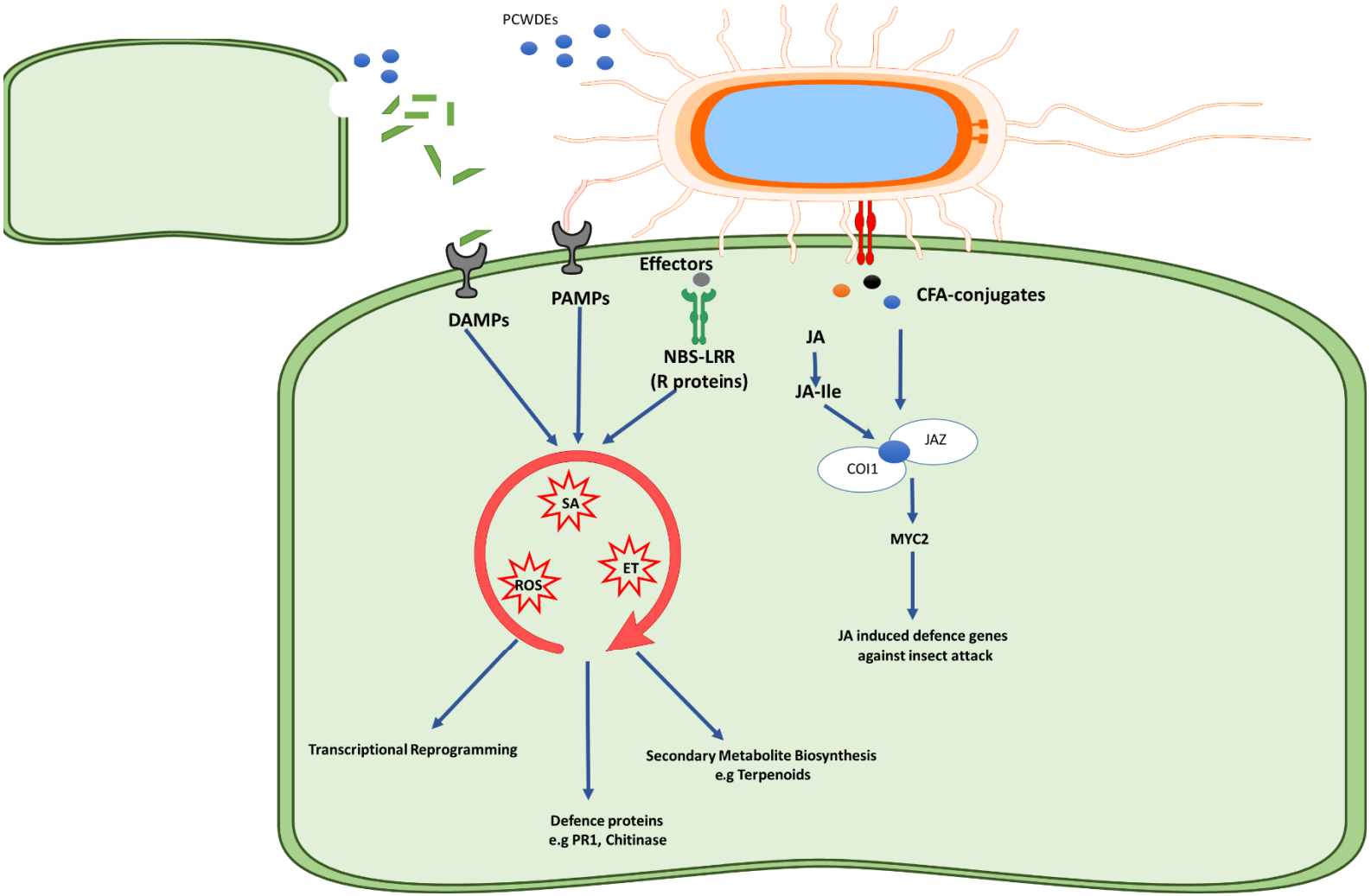


Figure 7.1: Component of DAMP-mediated PTI response in potato tuber 'Summer Delight' during *Pectobacterium* infection and treatment with exogenous Coronafacic acid.

Appendix A

A comparison of two software packages for analysing the transcriptional response of potato to *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* subsp. *brasiliensis*

A.1 RNA-seq read counts for each total RNA sample and the percentage of reads mapping to the Potato genome or to the genomes of *Pba* SCRI1043 and *Pbr* ICMP19477.

Sample Name	Replicates	Sampling time	Raw read count	Read count after Quality check	Percentage of reads mapped to Potato genome	Percentage of reads mapped to the genome of either *Pba* SCRI1043 or *Pbr* ICMP19477
Pba SCRI1043	1	6	52,096,784	51,866,638	69.50%	0.04%
	2	6	54,919,256	54,684,614	66.40%	0.01%
	3	6	48,937,612	48,718,624	66.90%	0.01%
Pba SCRI1043ΔHAI2	1	6	49,962,132	49,761,054	74.30%	0.02%
	2	6	52,185,162	51,993,160	69.90%	0.02%
	3	6	52,127,208	51,896,906	63.80%	0.17%
Pbr ICMP19477	1	6	52,285,618	52,070,636	74.40%	0.05%
	2	6	50,102,826	49,879,246	66.40%	0.33%
	3	6	48,222,800	48,010,476	74.00%	0.08%
CFA (200 nM)	1	6	48,364,490	48,161,008	69.80%	0.01%
	2	6	46,052,980	45,850,116	61.80%	0.01%
	3	6	49,820,988	49,623,568	67.00%	0.02%
$MgCl_2$ (10 mM)	1	6	47,479,098	47,307,918	67.80%	0.01%
	2	6	47,020,068	46,812,846	65.50%	0.06%
	3	6	47,266,078	47,076,372	68.70%	0.01%
Pba SCRI1043	1	12	48,138,814	47,927,876	67.80%	0.07%
	2	12	47,914,952	47,708,420	70.90%	0.02%
	3	12	44,858,980	44,670,778	65.40%	0.02%
Pba SCRI1043ΔHAI2	1	12	37,964,032	37,796,746	71.70%	0.02%
	2	12	43,320,974	43,150,332	62.20%	0.01%
	3	12	41,455,348	41,306,736	68.80%	0.02%
Pbr ICMP19477	1	12	45,136,766	44,940,314	73.60%	0.10%
	2	12	47,237,834	47,052,282	70.90%	0.04%
	3	12	46,975,554	46,769,946	72.20%	0.13%
CFA (200 nM)	1	12	48,401,996	48,193,246	66.20%	0.03%

	2	12	50,537,260	50,326,728	72.60%	0.01%
	3	12	44,860,132	44,663,800	71.50%	0.02%
	1	12	43,971,312	43,798,616	71.00%	0.01%
$MgCl_2$ (10 mM)	2	12	43,801,572	43,646,536	69.30%	0.03%
	3	12	40,841,350	40,663,044	67.20%	0.05%
	1	24	44,263,252	44,088,216	74.70%	0.01%
Pba SCRI1043	2	24	39,443,958	39,272,806	65.70%	0.05%
	3	24	43,461,184	43,274,746	56.10%	0.01%
	1	24	46,013,762	45,821,992	74.10%	0.02%
Pba SCRI1043ΔHAI2	2	24	45,895,534	45,692,384	76.10%	0.02%
	3	24	51,235,830	51,038,002	75.70%	0.02%
	1	24	50,747,660	50,567,858	69.10%	0.05%
Pbr ICMP19477	2	24	43,829,940	43,642,170	73.70%	0.08%
	3	24	48,570,612	48,382,338	56.10%	0.05%
	1	24	44,583,518	44,397,796	72.10%	0.07%
CFA (200 nM)	2	24	50,797,804	50,572,744	71.30%	0.02%
	3	24	51,855,252	51,634,774	69.60%	0.04%
	1	24	45,333,350	45,132,822	69.30%	0.01%
$MgCl_2$ (10 mM)	2	24	47,313,314	47,125,258	74.20%	0.02%
	3	24	47,842,250	47,666,266	66.10%	0.01%
	1	24	45,174,618	44,974,320	62.00%	0.03%
Non-inoculated control	2	24	42,802,756	42,630,256	72.80%	0.01%
	3	24	47,212,472	47,004,382	65.00%	0.04%

A.2 A list of the top 20 most DEGs observed in response to *Pbr* ICMP19477 using Cuffdiff2 analysis.

Gene I D	Gene Name	Log2 fold change	Raw read count data					
			Pbr	*Pbr*	*Pbr*	NI	NI	NI
PGSC0003DMG400002899	AP2/ERF domain-containing transcription factor	6.14	0.4	0.5	22.2	0.2	0.1	0.1
PGSC0003DMG400002993	Regulator of gene silencing	5.73	0.6	1.3	25.0	0.2	0.6	0.0
PGSC0003DMG400024003	FAD binding domain containing protein	5.26	0.5	5.8	0.2	0.1	0.0	0.0
PGSC0003DMG400002994	Regulator of gene silencing	4.48	2.2	2.1	20.5	0.1	1.1	0.0
PGSC0003DMG400014959	Acyltransferase	4.37	89.8	19.4	18.0	4.9	1.5	0.0
PGSC0003DMG400040538	Regulator of gene silencing	4.34	13.9	18.8	78.9	1.3	4.0	0.2
PGSC0003DMG400026046	Ethylene-responsive transcription factor 13	4.34	35.8	13.0	11.3	1.9	1.3	0.0
PGSC0003DMG400006462	GDSL-like Lipase/Acylhydrolase family protein	4.28	1.0	4.9	0.4	0.1	0.2	0.1
PGSC0003DMG400010639	S-adenosylmethionine-dependent methyltransferase	3.93	10.3	12.1	7.7	0.6	2.7	0.2
PGSC0003DMG402024842	Conserved gene of unknown function	3.80	35.3	7.0	5.5	1.7	1.9	0.1
PGSC0003DMG400007375	Chlorophyll a/b-binding protein PS II-Type I	-7.50	0.3	0.2	0.2	28.4	26.6	61.5
PGSC0003DMG400011740	SGA	-5.34	0.0	0.0	0.2	6.1	0.3	2.4
PGSC0003DMG401025899	Serine carboxypeptidase 1	-5.26	0.2	0.0	0.9	12.6	6.9	26.9
PGSC0003DMG400011752	Cellulose synthase	-5.05	0.0	0.0	0.1	3.9	0.8	2.5
PGSC0003DMG401011297	Gene of unknown function	-4.48	1.0	1.3	0.3	13.0	22.6	26.7
PGSC0003DMG402000216	Long-chain acyl-CoA synthetase 4	-4.41	0.1	0.0	0.1	4.7	0.2	0.4
PGSC0003DMG400032249	Non-specific lipid-transfer protein	-4.33	0.1	0.2	0.0	4.8	0.2	0.2
PGSC0003DMG400019323	Non-specific lipid-transfer protein	-4.30	0.1	0.0	0.3	8.6	0.5	1.1
PGSC0003DMG401007635	Gene of unknown function	-4.29	0.3	1.1	0.2	10.8	6.8	15.3
PGSC0003DMG400031731	Feruloyl transferase	-4.28	0.2	0.1	0.6	17.8	1.0	2.0

Numbers represent the log2fold change for each gene reported by Cuffdiff2 and the corresponding FPKM data obtained using Cufflinks analysis.

A.3 A list of the top 20 most DEGs observed in response to *Pbr* ICMP19477 using DESeq2 analysis.

Gene I D	Gene Name	Log2 fold change	Raw Read count data					
			Pbr	***Pbr***	***Pbr***	**NI**	**NI**	**NI**
PGSC0003DMG400023235	Sn-2 protein	6.09	53	450	311	1	1	0
PGSC0003DMG400012237	MYC2	4.97	87	93	111	0	1	0
PGSC0003DMG402002121	Glycine-rich protein	4.47	19	51	39	0	0	0
PGSC0003DMG400011441	Protein phosphatase 2c	3.69	38	39	19	0	0	0
PGSC0003DMG400015344	White-brown-complex ABC transporter family	3.54	28	29	50	1	2	0
PGSC0003DMG400030382	Class III peroxidase	3.5	34	58	28	0	0	0
PGSC0003DMG400017233	Ethylene-responsive transcription factor	3.42	46	29	68	0	0	0
PGSC0003DMG400011328	Mads box protein	3.34	34	26	32	3	1	0
PGSC0003DMG400002272	Transcription factor AP2-EREBP	3.24	98	27	57	2	0	0
PGSC0003DMG400013414	Chlorophyll a-b binding protein 3C, chloroplastic	-3.92	1	0	1	45	29	68
PGSC0003DMG401020908	Plasma intrinsic protein 2,1	-3.97	1	1	1	55	87	68
PGSC0003DMG400019217	AN2	-3.99	1	1	2	113	41	118
PGSC0003DMG400016068	Major latex	-4.15	1	1	2	228	36	118
PGSC0003DMG401001903	Cytochrome P450	-4.19	0	0	0	13	26	100
PGSC0003DMG400013416	Chlorophyll a-b binding protein 3C, chloroplastic	-4.21	0	0	1	32	53	59
PGSC0003DMG400011294	MYB transcription factor MYB114	-4.25	9	8	6	191	94	480
PGSC0003DMG400011052	Zinc finger protein	-4.36	1	0	2	39	54	71
PGSC0003DMG400009017	Stachyose synthase	-4.36	5	0	1	243	81	48
PGSC0003DMG401013418	Chlorophyll a-b binding protein 3C, chloroplastic	-5.2	0	0	0	77	67	134

Numbers represent the log2fold change for each gene reported by DESeq2 and the corresponding raw count obtained from the HT-seq count data.

Appendix B

Differential expression in potato tubers in response to *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* subsp. *brasiliensis*

B.1 A complete list of DEGs observed in response to *Pba* SCRI1043, *Pbr* ICMP19477 and mock-inoculation across all sampling time.

https://figshare.com/s/f1985906ea803abb1ba8

B.2 A list of commonly DEGs in response to *Pba* SCRI1043, *Pbr* ICMP19477 and the mock-inoculation across all sampling time.

https://figshare.com/s/1b33884b4502ae198b50

B.3 A list of commonly DEGs only in response to *Pba* SCRI1043 and *Pbr* ICMP19477 across all sampling time.

https://figshare.com/s/15d71ae2a8657e16e8b0

B.4 Gene ontologies of all DEGs observed in response to *Pba* SCRI1043 and *Pbr* ICMP19477 across all sampling time.

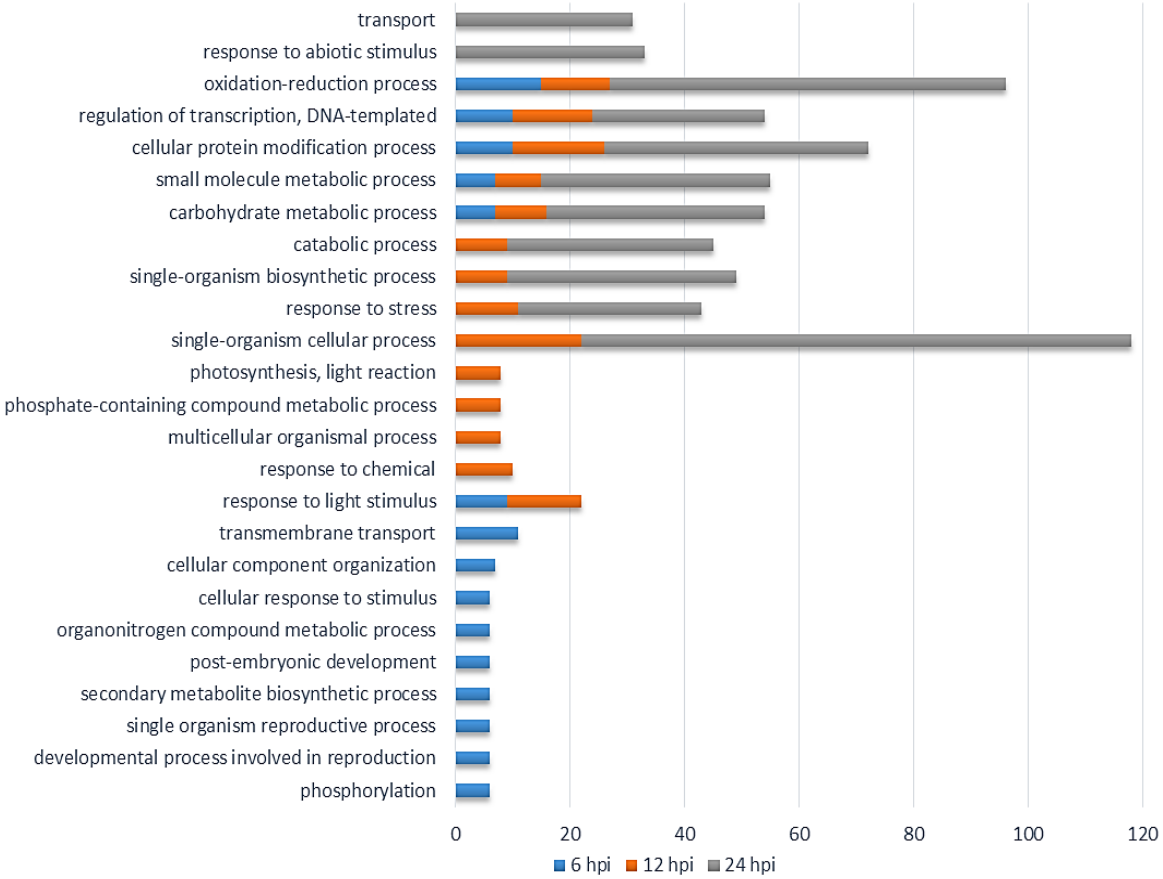


Figure B.4.1: Gene ontologies of all DEGs involved in the "biological processes" in response to *Pba* SCRI1043 across all sampling time.
BLAST, InterPro scan, and GO annotation implemented in BLAST2GO (3.3.5) were used to construct functional categories for DEGs. X axis represents the percentage of genes present in each category.

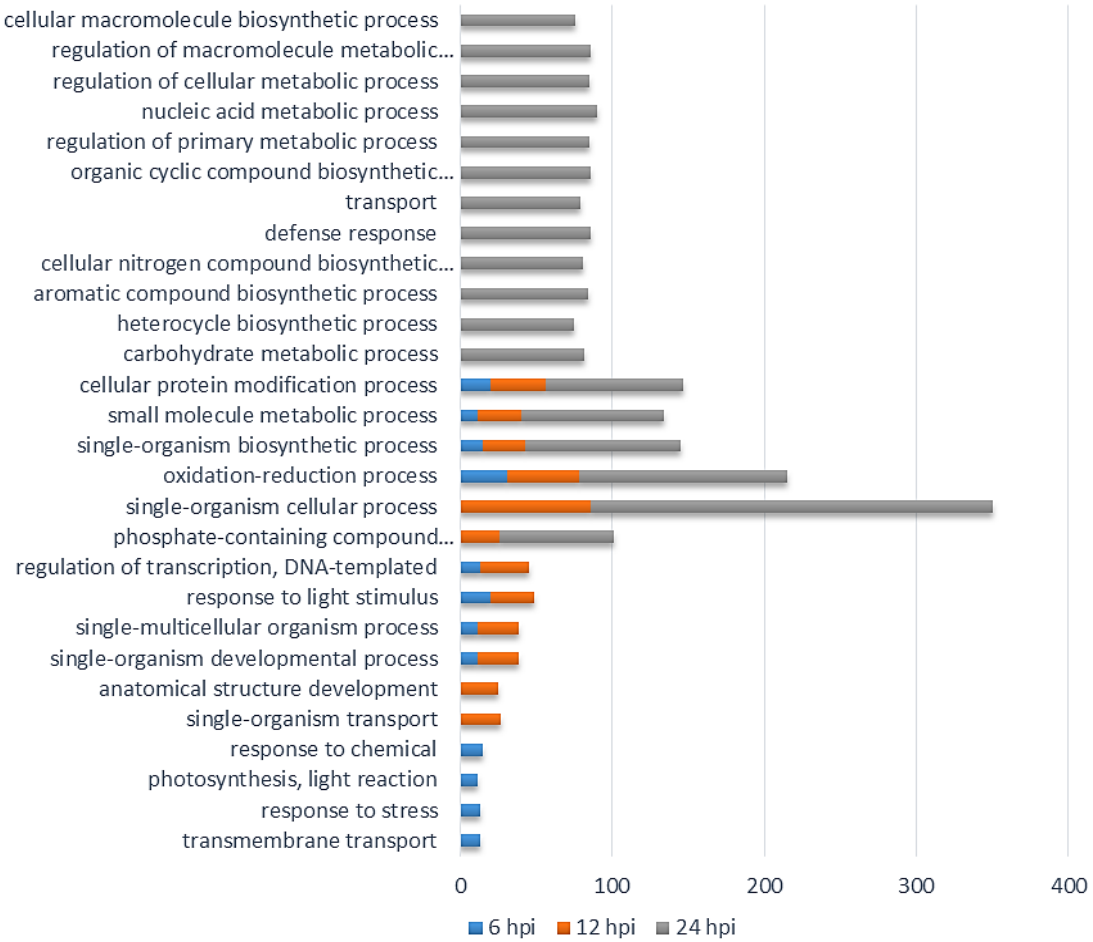


Figure B.4.2: Gene ontologies of all DEGs involved in the "biological processes" in response to *Pbr* ICMP19477 across all sampling time.
BLAST, InterPro scan, and GO annotation implemented in BLAST2GO (3.3.5) were used to construct functional categories for DEGs. X axis represents the percentage of genes present in each category.

B.5 A list of DEGs in response to *Pba* SCRI1043 and *Pbr* ICMP19477 when normalised with mock-inoculation control.

https://figshare.com/s/ade72d43b9593051bbf6

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